RecQ family helicases play a key role in chromosome maintenance. Despite extensive biochemical, biophysical, and structural studies, the mechanism by which helicase unwinds double-stranded DNA remains to be elucidated. Using a wide array of biochemical and biophysical approaches, we have previously shown that the_Escherichia coli_ RecQ helicase functions as a monomer. In this study, we have further characterized the kinetic mechanism of the RecQ-catalyzed unwinding of duplex DNA using the fluorometric stopped-flow method based on fluorescence resonance energy transfer. Our results show that RecQ helicase binds preferentially to 3’-flanking duplex DNA. Under the pre-steady-state conditions, the burst amplitude reveals a 1:1 ratio between RecQ and DNA substrate, suggesting that an active monomeric form of RecQ helicase is involved in the catalysis. Under the single-turnover conditions, the RecQ-catalyzed unwinding is independent of the 3’-tail length, indicating that functional interactions between RecQ molecules are not implicated in the DNA unwinding. It was further determined that RecQ unwinds DNA rapidly with a step size of 4 bp and a rate of ~21 steps/s. These kinetic results not only further support our previous conclusion that _E. coli_ RecQ functions as a monomer but also suggest that some of the Superfamily 2 helicases may function through an “inchworm” mechanism.

Helicases are molecular motor proteins that use the energy of nucleotide triphosphate hydrolysis to translocate along and separate the complementary strands of a nucleic acid duplex. These enzymes play essential roles in most aspects of the DNA metabolic pathway, such as replication, repair, recombination, and transcription (1–5). A large number of helicases have been identified; however, the mechanisms by which helicases unwind double-stranded DNA remain obscure.

One of the major concerns in studying the unwinding mechanism of a DNA helicase is its oligomeric state. This is because an oligomeric structure could provide multiple potential binding sites for the DNA substrate and nucleotide cofactors, which are absolutely required for the helicase to translocate along the DNA track during the processive DNA unwinding. Indeed, the biochemical and structural studies have shown that some helicases assemble into stable cooperative hexameric rings (3). These helicases include the E. _coli_ DnaB (6, 7) and Rho (8, 9), bacteriophage T4 gp41 (10, 11), and T7 gp4 (12–14). DNA passes through such a central channel and is unwound with a high processivity. For the non-ring helicases, the enzymes could be assembled into oligomers, and the DNA binding sites may be located on the separate subunits. On the basis of quantitative analyses of DNA binding properties, a “rolling model” was proposed to explain factorial DNA unwinding (15). This model suggests that each monomer of the Rep dimer binds alternatively to ssDNA and dsDNA. This process is regulated by repeated binding and hydrolysis of ATP and release of ADP. In this way, Rep dimers roll along the DNA with translocation coupled to ATP binding and DNA unwinding coupled to ATP hydrolysis. Kinetic studies have shown that both Rep (16, 17) and UvrD (18) helicases function as a dimer.

Whether a helicase can function as a monomer has attracted great attention. Based on the crystal structures of the Bacillus _stearothermophilus_ PcrA in the presence and absence of the nucleotides and DNA (19, 20), Wigley and co-workers (20–24) have found that the PcrA helicase functions as a monomer and that the structural and biochemical properties of the PcrA helicase are best explained by the “inchworm” mechanism. Consistent with this model, other helicases, such as UvrD (25), RecG (26), Dda (27, 28), RecQ (29), BLM (30), WRN (31), and NS3h (32), could also function as a monomer. The crystal structures of NS3h (33), RecG (34), Rep (35), and _E. coli_ RecQ (36) have revealed that these helicases are in the monomeric form. It is important to use other biochemical and biophysical methods to examine the oligomeric state of a helicase in order to gain a comprehensive understanding of the mechanism by which the helicase unwinds duplex DNA.

In this study, we focus on the kinetic DNA-unwinding mechanism and active oligomeric state of the _E. coli_ RecQ, a Superfamily 2 helicase. The RecQ family proteins are the conserved helicase enzymes involved in several aspects of DNA metabolism (37, 38). Three of human RecQ helicases, WRN (39), BLM (40), and RECQ4 (41), are associated with human genetic diseases, Werner, Bloom, and Rothmund-Thomson syndromes, respectively. The _E. coli_ RecQ, composed of 610 amino acids, is the prototype helicase of this family. The DNA unwinding studies of this enzyme have shown that the unwinding amplitude displays a sigmoidal dependence on ATP concentration with a Hill number of 3.3 (42). This observation has been taken as an argument that RecQ functions as a hexamer. However, we have previously shown that the RecQ helicase functions as a monomer (29), using an array of biochemical and biophysical approaches. Our studies employed size exclusion...
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TABLE 1
Substrates used in DNA binding and unwinding assays

| Substrate | Structure and sequence |
|-----------|------------------------|
| 16-bp duplex with 3’-ssDNA tail | 3’-
| 16-bp duplex with 5’-ssDNA tail | 5’-
| 12-bp duplex with 10-nt 3’-ssDNA tail | 3’-
| 20-bp duplex with 10-nt 3’-ssDNA tail | 5’-
| 25-bp duplex with 10-nt 3’-ssDNA tail | 3’-
| 30-bp duplex with 10-nt 3’-ssDNA tail | 5’-
| 35-bp duplex with 10-nt 3’-ssDNA tail | 3’-
| 40-bp duplex with 10-nt 3’-ssDNA tail | 5’-

* F, fluorescein.
* H, hexachlorofluorescein.

chromatography, analytical ultracentrifugation, time-resolved fluorescence anisotropy, and functional analysis. Our conclusion is consistent with the crystal structure of the RecQ in the apo form (36). However, several questions related to the oligomeric state of the active RecQ helicase remain to be addressed. First, the oligomers could be transient in nature or unstable to be detected by these approaches. Second, the crystal structure of RecQ was obtained with the C-terminal truncated protein, in the absence of a DNA substrate (36). The oligomeric state of the truncated protein in the absence of DNA could be different from that of the full-length protein in the presence of DNA. Furthermore, the full-length BLM (43) and the exonuclease domain of WRN (44) form oligomeric ringlike structures in solution, revealed by electron microscopy and atomic force microscopy, respectively. As the RecQ family helicases are highly conserved in sequence and in function, it is therefore possible that the RecQ helicase could also function as a hexamer in solution.

In this study, we resorted to the rapid stopped-flow fluorescence assays that were based on the fluorescence resonance energy transfer, allowing the continuous and real-time observations of the DNA unwinding (16, 45). By taking advantage of these approaches, the RecQ-mediated DNA unwinding kinetics was analyzed under both pre-steady-state and single-turnover DNA unwinding conditions. Our results support our previous conclusion that RecQ is a monomeric DNA helicase.

MATERIALS AND METHODS

Reagents and Buffers—All chemicals were reagent grade, and all buffers were prepared in high quality deionized water from a Milli-Q® ultra-pure water purification system (Millipore) having resistivity greater than 18.2 megaohms-cm. All unwinding reactions and DNA binding assays were performed in buffers containing 25 mM Tris-HCl (pH 7.4 at 25 °C), 50 mM NaCl, 1 mM MgCl2, and 0.1 mM dithiothreitol unless noted elsewhere. ATP was from Sigma and dissolved as a concentrated stock at pH 7.0. ATP concentration was determined by using an extinction coefficient at 260 nm of 1.48 × 104 M cm−1. ATPase activity was measured by the method of Chmiel and Brosius (27). The protein concentration was determined by the method of Lowry et al. (28) with bovine serum albumin as the standard. All synthetic oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before storage in 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol at −80 °C.

Fluorophores—Fluorescein isothiocyanate (FITC) and hexachlorofluorescein (HCF) were from Molecular Probes. RecQ protein was stored in storage buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 3 mM MgCl2, and 2 mM dithiothreitol) at −80 °C.

Oligonucleotide Reaction Substrates—Substrates were synthesized according to the DNA substrates used in the unwinding assays. All substrates were labeled with fluorescein and hexachlorofluorescein, respectively, whereas those used in the DNA binding assays have only one strand labeled with fluorescein. Their sequences, similar to those in our previous studies (46), are shown in Table 1. The protein trap used for single-turnover kinetic experiments was 56-nt poly(dT). ATP, single-stranded oligonucleotides, with or without fluorescent labels, were purchased from SBS Genetec Co., Ltd. (Beijing, China), and all synthetic oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before storage in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at −20 °C. Concentrations of single-stranded oligodeoxyribonucleotides were determined spectrophotometrically based on extinction coefficients calculated by the nearest neighbor method. A 50 μM working stock of dsDNA was prepared by mixing equal concentrations of complementary single-stranded oligonucleotides in a 20 mM Tris-HCl buffer (pH 7.5 at 25 °C) containing 100 mM NaCl, followed by heating to 85 °C. After equilibrating for 5 min, annealing was allowed by slow cooling to room temperature. The duplex was stored at −20 °C. In order to evaluate the efficiency of the oligonucleotide hybridization, the 3’-fluorescein-labeled ssDNA was further labeled with 10P at its 5’-end. The hybridization was then performed under the same conditions as above. The resulting dsDNA and ssDNA were separated by native gel and further quantified by PhosphorImager analysis. The results showed that the quantity of ssDNA represents 0.5–2%.

Fluorescence Anisotropy Measurements—DNA Binding of RecQ was analyzed by fluorescence polarization as described previously (46, 48). The assays were performed using a Bio-Logic autotitrator (TUC-250) and a Bio-Logic optical system (MOS450/AF-CD) in fluorescence anisotropy mode. Varying amounts of proteins were added to 1 ml of binding buffer containing 5 mM DNA substrate. Each sample was allowed to equilibrate in solution for 1.5 min, after which fluorescence polarization was measured. Titrations were performed in a temperature-controlled cuvette at 25 °C. The solution was stirred continuously by a small magnetic stir bar during the whole titration process.

The stoichiometry (N) and dissociation constant (K_D) in units of molarity were determined by globally fitting the anisotropy data according to Equation 1 (49, 50),

\[
\alpha D_f = \frac{N \alpha}{1 - \alpha} - K_D
\] (Eq. 1)
where $D_T$ represents the total concentration of DNA and $P_T$ is the concentration of RecQ protein in the binding solution, and Equation 2,

$$\alpha = \frac{A_{\text{max}} - A}{A_{\text{max}} - A_{\text{min}}} \quad \text{(Eq. 2)}$$

where $A$ is the fluorescence anisotropy at a given concentration of RecQ, $A_{\text{max}}$ is the anisotropy at saturation, and $A_{\text{min}}$ is the initial anisotropy. For convenience of fitting, Equation 1 can be further written as follows,

$$A = A_{\text{min}} + \left(\frac{A_{\text{max}} - A_{\text{min}}}{\Delta - 4c_vD_TNPT}\right) \Delta^2 - 4c_vD_TNPT$$

(Eq. 3)

where $\Delta = c_vD_T + NPT$ and $c_v$ is used to account for the decrease of DNA concentration during the titration. In terms of the RecQ concentration in the binding solution ($P_r$) and that in the titrant ($P_{\text{titrant}}$), $c_v$ can be expressed as $c_v = 1 - \frac{P_r}{P_{\text{titrant}}}$. 

**Stopped-flow Fluorescence Measurements**—The stopped-flow assays were carried out using a Bio-Logic SFM-400 mixer with a 1.5 mm × 1.5 mm cell (FC-15, Bio-Logic) and the Bio-Logic MOS450/AF-CD optical system equipped with a 150-watt mercury-xenon lamp. Fluorescein was excited at 492 nm (2-nm slit width), and its emission was monitored at 525 nm using a high pass filter with 20-nm bandwidth (D525/20; Chroma Technology Co.). Unwinding kinetics were measured in a two-syringe mode, where RecQ helicase and duplex DNA substrates were preincubated at 25 °C in syringe 1 for 5 min while ATP and protein trap were in syringe 4. Each syringe contained unwinding reaction buffer, and the unwinding reaction was initiated by rapid mixing. All concentrations listed are after mixing unless noted otherwise. For converting the output data from volts to percentage unwinding, a calibration experiment was performed in a four-syringe mode, where helicase 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 of the solutions were filtered and extensively degassed immediately before they were used. The stopped-flow temperature was controlled by means of an external thermostated water bath (B12; Thermo Haake) and a high flux pump to circulate the water between the bath and the stopped-flow apparatus. The standard reaction temperature was 25 °C.

**Kinetic Data Analysis**—As indicated under “Results,” all stopped-flow kinetic traces were an average of over 15 individual traces. The kinetic traces were analyzed using Bio-Kine (version 4.26; Bio-Logic) with one of the following equations.

$$A(t) = A(1 - e^{-k_{\text{obs}}t}) \quad \text{(Eq. 4)}$$

$$A(t) = A_1(1 - e^{-k_{\text{obs}}t}) + A_2(1 - e^{-k_{\text{obs}}t}) \quad \text{(Eq. 5)}$$

$$A(t) = A_1(1 - e^{-k_{\text{obs}}t}) + k_{\text{steady}}t \quad \text{(Eq. 6)}$$

$$A(t) = A_1 \left(1 - \sum_{n=1}^{\infty} \frac{k_{\text{obs}}}{(n-1)!} t^{n-1} e^{-k_{\text{obs}}t}\right) + k_{\text{steady}}t \quad \text{(Eq. 7)}$$

In Equation 4, $A(t)$ represents the unwinding amplitude as a function of time, $A$ is the total unwinding amplitude, and $k_{\text{obs}}$ is the unwinding rate constant. In Equation 5, $A_1$ and $A_2$ represent, respectively, the unwinding amplitude and rate of the fast (slow) phase. In Equation 6, $A_1$ and $k_{\text{obs}}$ represent, respectively, the unwinding amplitude and rate of the fast phase, and $k_{\text{steady}}$ represents the steady-state rate. It should be noted that Equation 6 corresponds to Equation 5 (i.e. $k_{\text{steady}} = A_1/k_{\text{obs}}$) in the special case of $k_{\text{obs}}t \ll 1$.

Equation 7 is used to determine the step size of DNA unwinding (51, 52), where $A_1$ is the unwinding amplitude of the fast phase, $n$ is the number of steps required to unwind the duplex DNA, and $k_{\text{obs}}$ is equivalent to the unwinding rate in one step when neglecting the dissociation rate of the enzyme during unwinding.

**RESULTS AND DISCUSSION**

RecQ Preferentially Binds 3′-Flanking Duplex DNA in Proportion to ssDNA Tail Length—It has been shown that RecQ helicase unwinds dsDNA in the 3′–5′ direction and binds both ssDNA and dsDNA with a preference for ssDNA (46, 48, 53). However, the binding affinity of RecQ for a partial dsDNA that contains a 3′- or 5′-tail, compared with ssDNA and dsDNA, is not known, and neither is the binding preference for 3′- or 5′-overhanded dsDNA. To address these questions, the equilibrium dissociation constants ($K_c$) of the RecQ helicase for three different DNA substrates were determined using a fluorescence polarization assay, as described previously (46, 48). The blunt-ended and 3′- or 5′-overhanded dsDNA were used. The titration experiments were performed under conditions essentially identical to those used for the DNA unwinding, but without ATP, by titrating 5 nM of dsDNA (initial concentration) with increasing concentrations of RecQ. The anisotropy of the fluorescein-labeled DNA was then measured. The fluorometric data were fitted to Equation 3 to determine the stoichiometric number ($N$) and the dissociation constant.

The typical titration curves are given in Fig. 1A. RecQ did not bind the blunt-ended dsDNA until the concentration reached 150 nM. However, RecQ did bind the 3′-overhanded dsDNA with a 5′–50 nt tail. RecQ could also bind 5′-overhanded dsDNA but with a much lower affinity (see Fig. 1C). The stoichiometric numbers and dissociation constant were then determined using Equation 3. For the 3′-overhanded dsDNA, the number of the RecQ molecules bound per DNA substrate increased linearly with increasing tail length, with a slope of 0.114 ± 0.003 RecQ molecule/nt (Fig. 1B), which corresponds to a binding size of 8.8 ± 0.2 nt. This number is similar to that determined previously (10 nt) (48). The dissociation constant per site, $K_c/N$, for both 3′- and 5′-overhanded dsDNA was given in Fig. 1C. The lowest $K_c/N$ values were observed for the 3′-overhanded dsDNA with a 3′-ssDNA tail of 10 and 15 nt, indicating that the RecQ protein had a much higher affinity for binding to the ss/dsDNA junction than to ssDNA. This explains why RecQ could still bind very well to the ss/dsDNA substrate with only a 5-nt tail, whereas it had very low affinity even for a 7-nt ssDNA substrate (48). Similar phenomena have been recently observed for NS3h (54). The noncooperative binding model (Equation 3) fitted well to the binding curves, indicating that RecQ binds DNA with no cooperativity. This is in agreement with the previous observation (48). These results indicate that the RecQ helicase preferentially binds the 3′-overhanded dsDNA.

Pre-steady-state Kinetic Analysis of DNA Unwinding Catalyzed by RecQ Reveals That the Enzyme Functions as a Monomer—Pre-steady-state kinetic analysis has been shown to be a powerful method of determining the quantity of active enzyme in the reaction mixture by measuring the first cycle of product formation. Knowledge of the stoichiometry between the formed product and the active enzyme involved in the catalysis can offer an insight into the mechanism by which the enzyme functions (55). Recently, in combination with
Based on the DNA binding behaviors of RecQ as determined above, we had chosen the 16-bp dsDNA with a 10-nt ssDNA tail as the substrate, because under these conditions, RecQ is bound to this substrate with a stoichiometry of one RecQ molecule per DNA substrate (Fig. 1B). To observe a burst of unwinding reaction in the pre-steady-state assay, the concentration of the DNA substrate must exceed that of the enzyme as well as the value of $K_D$, such that the majority of RecQ will bind to the substrate before initiation of the unwinding reaction (28). The dissociation constant $K_D$ of RecQ from the 10-nt-tailed substrate was determined to be $5.5 \pm 0.5$ nM (Fig. 1C). Thus, we first used 20 nM DNA and 4-fold less RecQ (5 nM) for the initial pre-steady-state unwinding experiments. The excess ssDNA/dsDNA substrate was first preincubated with RecQ helicase for 5 min; the reaction was then initiated by the rapid addition of 1 mM ATP and observed continuously by monitoring the fluorescence enhancement of fluorescein due to the RecQ-catalyzed strand separation.

Under these conditions, the kinetics of the pre-steady-state unwinding reaction, averaged over 15 experiments, was biphasic (Fig. 2A). The first phase (i.e. the burst phase) represents the first cycle of the unwinding reaction, whereas the second phase represented the steady-state reaction. The data can be well fitted with a single exponential followed by a steady-state rate (Equation 6). The burst amplitude was $4.6 \pm 0.1$ nM, which was very close to the concentration of RecQ (5 nM). This implies that RecQ functions as a monomer and can effectively unwind the 16-bp dsDNA with a sufficient processivity. The rate constant in the burst phase was $7.8 \pm 0.1$ s$^{-1}$. This corresponds to an unwinding rate of 124.8 bp s$^{-1}$, indicating that RecQ is a rapid and monomeric helicase.

To examine whether the steady-state phase of RecQ-catalyzed unwinding under pre-steady-state conditions affects the burst phase, we performed a control experiment under the same conditions as described above except that a protein trap (dT$_{56,2}$,2$_{56,2}$) was added at the same time as ATP. The excessive protein trap prevented, at the beginning of as well as during the unwinding reaction, any free RecQ molecules from rebinding to duplex DNA substrates. As shown in Fig. 2B, the steady-state phase of the reaction was totally eliminated, whereas the amplitude ($4.4 \pm 0.1$ nM) and the rate ($8.4 \pm 0.1$ s$^{-1}$) of the burst phase was very similar to that in the previous case of no protein trap. This indicates that the burst phase of the unwinding reaction under pre-steady-state conditions without protein trap produces reliable information on the first cycle of reaction.

To determine whether oligomerization of RecQ occurs at a higher ratio of protein to DNA, we performed a series of RecQ-catalyzed unwinding experiments under the pre-steady-state conditions, using the concentrations of RecQ from 5 to 40 nM but maintaining the concentration of the 10-nt-tailed ssDNA/dsDNA constant (60 nM). The results of these experiments are shown in Fig. 2C. The data at low RecQ concentrations could still be described well with a single exponential followed by a steady-state rate, whereas those at high RecQ concentrations could only be described well with a double exponential (Equation 5). To be consistent, we fitted all of the data according to Equation 5, in which $A_1$ and $k_{obs,1}$ correspond to the amplitude and rate of the burst phase, and $A_2$ and $k_{obs,2}$ corresponds to the initial unwinding rate of the second phase and is equivalent to the steady-state rate at low RecQ concentrations.

As in the previous experiment (Fig. 2A), the burst amplitude of the unwinding reaction was close to the RecQ concentration for each case in Fig. 2C. The ratio of the burst amplitude over RecQ concentration remained high and invariable (averaged $0.80 \pm 0.06$) in the whole range of [RecQ] (Fig. 3A). In addition, the rate of the burst phase was also similar in the whole range (averaged $9.0 \pm 0.6$ s$^{-1}$) (Fig. 3B). These results clearly show that the helicase activity of RecQ does not change at
the higher concentration, suggesting that the dsDNA substrate is unwound by a single RecQ molecule under the condition. The initial rate of the second phase, $A_2 k_{obs,2}$, is given in Fig. 3C. It increases linearly with the RecQ concentration. Since the second phase reflects the helicase activity of dissociated RecQ molecules after initiation of the reaction, the linear relation in Fig. 3C also indicates that the behavior of the "steady-state" helicase activity does not vary with the protein concentration in the range tested.

Since the above experiments were performed with 10-nt-tailed ssDNA/dsDNA, the conditions under which only one enzyme molecule could bind to the DNA substrate, we therefore conclude that the RecQ helicase rapidly and efficiently unwinds dsDNA as a monomer.

Single-turnover Kinetics Study Shows That DNA Unwinding Efficiency Is Independent of the ssDNA Tail Length, Further Confirming That RecQ Is Active as a Monomer—The above results clearly show that the RecQ helicase functions as a monomer. However, it remains to be seen whether functional interactions between the protein molecules could enhance the DNA unwinding efficiency. To answer this, we then examined the single-turnover kinetics of DNA unwinding of 16-bp dsDNA with a 3'-ssDNA tail length from 5 to 50 nt. We reasoned that,
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if RecQ always functioned as a monomer, it should be capable of unwinding all of the different substrates with similar unwinding rates, regardless of the tail length. Otherwise, if functional interactions are involved in the DNA unwinding, the unwinding rate should be dependent on the tail length of the substrate.

To ensure single-turnover conditions, the fluorescence stopped-flow experiments were performed by preincubating a 2 nM concentration of the DNA substrate with 50 nM of RecQ for 5 min. Under these conditions, the RecQ molecules should saturate the DNA substrates. The unwinding reaction was then initiated by adding 1 mM ATP and an excess of protein trap (dT$_{50}$, 2 μM). The excessive protein trap was used to prevent any free RecQ molecules from rebinding to dsDNA, as in the case of Fig. 2B, at the beginning of and during the unwinding reaction.

Under identical single-turnover conditions, we observed obvious unwinding for all of the DNA substrates with 3'-ssDNA tails varying from 5 to 50 nt. Typical results are given in Fig. 4. As observed previously (45), no unwinding was observed for the blunt-ended dsDNA under the same conditions (data not shown). For DNA substrates with short tails (5 and 10 nt), in which case only one RecQ monomer is expected to bind to each DNA substrate, the RecQ-catalyzed unwinding was rapid and efficient. The kinetics of unwinding were monophasic and the time courses could be fitted well to a single exponential. For DNA substrates with longer tails (>15 nt), the RecQ-catalyzed unwinding was also fast and efficient, but the kinetics of unwinding were biphasic, and the time courses could be fitted well only to a double exponential.

The observed unwinding rates obtained from the global analysis of the single-turnover kinetic courses were plotted as a function of the ssDNA tail length, as shown in Fig. 5A. The kinetic unwinding traces were quite different for short- and long-tailed DNA substrates. However, the observed rate constants for the only phase in the short-tailed case and for the fast phase in the long-tailed case were similar, with an average value of 7.3 ± 0.6 s$^{-1}$, which was close to the burst rate under the pre-steady-state conditions (9.0 ± 0.6 s$^{-1}$; Fig. 3B). This result indicates that the fast unwinding phase for DNA with a long ssDNA tail is due to RecQ monomer and that these monomers should be ones that are bound to the ssDNA/dsDNA junction. Since the slow unwinding phase is only observable with the long-tailed substrates, this kinetic behavior should be due to the trailing monomer. If the dissociation from the substrates occurred for those leading monomers upon initiation of unwinding, the trailing monomers would continue the unwinding but with a slower unwinding rate due to their translocation along the ssDNA tail toward the ssDNA/dsDNA junction. From the above considerations, we expect that whereas the amplitude of the fast phase should be independent of the ssDNA tail length, that of the slow phase should increase with the tail length because the number of monomers binding to the ssDNA tails is proportional to the ssDNA length.

As shown in Fig. 5B, the amplitude of the slow phase indeed increases with the increase of the ssDNA tail length and becomes saturated at a tail length of 30 nt, implying that the additional molecules on longer
ssDNA tails do not contribute to the unwinding. The amplitude of the fast phase, on the other hand, drops unexpectedly from ~0.75 to ~0.55 at a tail length of 20 nt rather than being independent of the tail length. The simplest explanation is that the leading monomers are bound to the ssDNA/dsDNA junctions more tightly on short-tailed substrates (5, 10, and 15 nt), due to limitation of available binding space, than on long-tailed substrate (Fig. 1C), and thus it is more difficult for them to dissociate from the short-tailed substrates upon initiation of the unwinding reaction. The dissociation rate during unwinding should be negligible for its high processivity. Note that the unwinding amplitude for the substrate with a 5-nt tail is as high as ~0.7, indicating that the 5-nt-tailed ssDNA/dsDNA substrate is still bound productively, consistent with the DNA binding measurements (Fig. 1). The total amplitude of the RecQ-catalyzed unwinding is shown in Fig. 5C. It slightly increases with the ssDNA tail length and then is saturated (~0.9). These results, taken together, further confirm our previous observations that RecQ helicase functions as a monomer and that the functional interactions between molecules are not necessary for efficient DNA unwinding. This is different from the case of NS3h, where, as evidenced by systematic single turnover unwinding kinetic studies (32), the functional interaction between the helicase molecules contributes to efficient DNA unwinding.

Step Size Analysis Reveals RecQ Having a High Mechanochemical Coupling Efficiency—Helicase functions as a molecular motor that converts chemical energy to mechanical motion for translocating along the nucleic acid track through consecutive (multiple) steps. Determination of the step size is necessary to fit the experimental data to a kinetic model, which relates the translocation rate constant to the number of steps (or step size and duplex length) (Equation 7) or alternately that given by Neuman et al. (56). We therefore determined the step size with a series of 10-nt-tailed ssDNA/dsDNA substrates varying in duplex length from 12 to 40 bp. The experiments were performed under conditions similar to those in the pre-steady-state studies (Fig. 2A). 20 nM ssDNA/dsDNA substrate was first preincubated with 5 nM RecQ helicase for 5 min, and the reaction was then initiated by the rapid addition of 1 mM ATP. Since the ssDNA tail length was 10 nt, only one RecQ molecule was expected to bind each DNA substrate.

As observed above, the kinetics of RecQ-catalyzed DNA unwinding are biphasic. We have observed that the unwinding amplitude decreases significantly for long dsDNA substrates. No unwinding was observed for substrates longer than 25 bp. The reason should be that, during the unwinding progress by a single RecQ molecule and with the length of the unwound DNA strand increasing, the strands can reanneal behind the unwinding complex rapidly. A similar phenomenon has been observed with NS3h helicase (32). Low unwinding processivity of a single molecule may also be responsible for a decrease of unwinding amplitude for long dsDNA substrates. However, this should not be the case for RecQ, which has a high processivity.

The unwinding curves for 12-, 16-, and 20-bp duplex substrates are given in Fig. 6A. The amplitude of the fast phase is quite similar for the 12- and 16-bp substrates, whereas that for the 20-bp substrate is significantly reduced due to reannealing of the long unwound ssDNA. Fitting these data to Equation 7, the numbers of steps for the different substrates were determined (Fig. 6B). The number of steps increases linearly with the dsDNA length with a slope of 0.25 ± 0.0 bp⁻¹, giving a step size of 4 bp. Since the average value of k₁₆, is 21.0 ± 1.0 s⁻¹, the unwinding rate of RecQ is 84 ± 4 bp s⁻¹. This value is much higher than the 1–3 bp s⁻¹ determined under steady-state conditions (29, 42, 46). This discrepancy may be mainly due to the different experimental conditions (under steady-state conditions in the previous studies and pre-steady-state conditions in this report) and different DNA substrates used. It is worth noting that the unwinding rate determined in this study is comparable with that determined from a single molecule study. It is highly possible that the unwinding rate determined previously by the non-stopped-flow methods underestimated the unwinding rate. By comparison with other helicases (Table 2), we conclude that RecQ is a rapid helicase.

**Conclusion**—Whether the active form of a helicase is oligomeric represents one of the central questions regarding helicases. The list of heli-

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**TABLE 2**

| Helicase | Unwinding rate (bp s⁻¹) | Unwinding rate (bp s⁻¹) | References | Comments |
|----------|-------------------------|-------------------------|------------|----------|
| PcrA     | 50–100                  | 1                       | 22, 57     | Measured with ssDNA translocation |
| NS3h     | 1.6                     | 9.2                     | 32         | Quenched flow |
| Rep      | 64                      | 4.5                     | 17         | Quenched flow |
| UvrD     | 86                      | 4.4                     | 51         | Quenched flow |
| RecBCD   | 680                     | 3.4                     | 59         | Stopped flow |
| NPH-II   | 13                      | 6                       | 62         | Quenched flow |
| RecQ     | 84                      | 4                       | Current work | Stopped flow |

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4 M.-N. Dessinges, T. Lionnet, X. G. Xi, D. Bensimon, and V. Croquette, unpublished observation.
cases that may function as monomers is lengthening. It includes the Superfamily 1 helicases, such as PcrA (21, 22), UvrD (25), and Dda (27, 28), and the Superfamily 2 helicases, such as RepC (26), BLM (30), WRN (31), and NS3h (32). Previously, we have shown, with a variety of methods, that the Superfamily 2 RepC helicase is monomeric in solution as well as during its catalysis (29). To further investigate its kinetic mechanism of DNA unwinding, we performed both pre-steady-state and single-turnover DNA unwinding kinetic analyses as described in this study. The pre-steady-state kinetic analysis of DNA unwinding catalyzed by RepC reveals that the enzyme functions as a monomer. The single-turnover kinetics study shows that DNA unwinding efficiency is independent of the ssDNA tail length, further confirming that RepC functions as a monomer. The unwinding step size is 4 bp, and the unwinding rate reaches as high as 84 bp s⁻¹, showing that the E. coli RepC unwinds DNA rapidly. This insight, together with the information available from the literature (Table 2), leads us to conclude that the E. coli RepC functions as a monomer and efficiently couples the mechanochemical force to a rapid DNA unwinding.

The structural bases of RepC helicase functioning as a monomer remain to be elucidated. To date, the information from the two structurally and biochemically well-characterized helicases, PcrA and Rep, suggest that some of the SF1 and SF2 helicases possess the intrinsic ability to function as a monomer. Based on the crystal structures of PcrA in the apo form and in a complex with a short ssDNA dsDNA substrate (19, 20), an inchworm mechanism was proposed to explain how the monomeric PcrA helicase functions (23). Subsequent biochemical characterization of the wild type and mutant PcrA helicase (24) suggests that the inchworm mechanism involves the destabilization of the dsDNA ahead of the enzyme that is coupled to the unidirectional DNA translocation along the single strand product.

Although Rep protein is in monomeric form in the crystal structure (35), extensive biochemical characterization of Rep protein has established a convincing conclusion that a Rep dimer is the minimal form required for helicase activity in vitro (17). This view is further supported by single molecule studies, which further reveal why Rep should function as a dimer (63). In fact, Rep helicase activity is highly regulated through intramolecular interaction: the 2B subdomain functions as a regulatory domain and inhibits the intrinsic helicase activity of the full-length Rep (Fig. 7A). Dimerization may alleviate the inhibitory effect of the 2B subdomain. Consistent with this proposal, the Rep helicase mutant in which the 2B subdomain is deleted (RepΔ2B) functions as a monomer and unwinds DNA more efficiently than the wild-type enzyme (64, 65). These observations indicate that Rep helicase possesses the intrinsic ability to function as a monomer. The 2B subdomain of Rep is located between motif IV and motif V (Fig. 7B). It contains none of the highly conserved helicase motifs that are absolutely required for helicase activity. A comparison of the amino acid sequence and the crystal structure of RepC with that of Rep indicates that an equivalent 2B subdomain is absent from RepC helicase (Fig. 7). Thus, RepC helicase is structurally more close to the RepΔ2B mutant, which functions more efficiently as a monomeric helicase than the full-length dimeric Rep helicase.

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Note Added in Proof—Interestingly, recent single molecular study (66) shows that monomeric Rep helicase does not unwind duplex DNA, but repetitively shuttles along the ssDNA region, possibly keeping the DNA clear of toxic recombination intermediates. Therefore, dimerization is not absolutely necessary for DNA unwinding as proposed by the rolling model, but turns shutting to DNA unwinding.

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