Bacillus stearothermophilus PcrA Monomer Is a Single-stranded DNA Translocase but Not a Processive Helicase in Vitro*5

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Structural studies of the Bacillus stearothermophilus PcrA protein along with biochemical studies of the single-stranded (ss) DNA translocation activity of PcrA monomers have led to the suggestion that a PcrA monomer possesses processive helicase activity in vitro. Yet definitive studies testing whether the PcrA monomer possesses processive helicase activity have not been performed. Here we show, using single turnover kinetic methods, that monomers of PcrA are able to translocate along ssDNA, in the 3' to 5' direction, rapidly and processively, whereas these same monomers display no detectable helicase activity under the same solution conditions in vitro. The PcrA monomer ssDNA translocation activity, although necessary, is not sufficient for processive helicase activity, and thus the translocase and helicase activities of PcrA are separable. These results also suggest that the helicase activity of PcrA needs to be activated either by self-assembly or through interactions with accessory proteins. This same behavior is displayed by both the Escherichia coli Rep and UvrD monomers. Hence, all three of these SF1 enzymes are ssDNA translocases as monomers but do not display processive helicase activity in vitro unless activated. The fact that the translocase and helicase activities are separable suggests that each activity may be used for different functions in vivo.

DNA helicases are nucleic acid motor proteins that use nucleoside triphosphate binding and hydrolysis to unwind duplex DNA (1–4). These enzymes generally also have the ability to translocate with biased directionality along single-stranded (ss)DNA (5–9). In fact, some of the biological activities of these enzymes, such as displacement of other proteins from ssDNA (10–16), may be dependent only on the ability of these enzymes to translocate along ssDNA rather than on helicase activity. DNA helicases have been grouped into families and superfamilies defined by conserved amino acid sequence motifs (17). These enzymes can also be divided into two distinct structural classes, with one class functioning as toroidal or ring-like hexamers (4). By far, the largest number of helicases and putative helicases belong to the nonhexameric SF1 or SF2 superfamilies (17).

SF1 enzymes have often been considered to be monomeric helicases (18, 19). Yet direct experimental tests of whether a monomeric protein has helicase activity have been performed for only a few enzymes. The most direct experimental approach to address the question of what oligomeric form of the enzyme is needed for helicase activity requires examination of the DNA unwinding kinetics under single turnover conditions, such that only a single round of DNA binding is allowed. In such an experiment, the DNA substrate is prebound with enzyme under conditions such that the oligomeric form of the enzyme is known (e.g. a monomer), and the reaction is started by the addition of nucleoside triphosphate (e.g. ATP) along with a “trap” for free enzyme (20, 21). Any DNA unwinding that is observed in such a single turnover experiment must be catalyzed by the form of the enzyme that was prebound to the DNA because the trap prevents additional enzyme from binding to the DNA. In contrast, multiple turnover DNA unwinding experiments have been the most common method used to examine helicase activity. Such multiple turnover kinetics experiments cannot provide an unambiguous answer to this question because enzyme dissociation and reassociation can occur during the reaction, and thus the oligomeric state of the enzyme involved in the unwinding process is not known with certainty and even can change during the multiple turnover reaction.

Using such single turnover DNA unwinding experiments, it has been shown that the SF1 enzymes E. coli Rep (22, 23) and E. coli UvrD (24–26) require oligomerization to function as helicases in vitro, even though monomers of both of these enzymes are able to translocate with 3' to 5' directionality along ssDNA rapidly and processively (7, 8, 23). On the other hand, the SF1 phage T4 Dda protein (27), the F episome Tra I protein (28), and the E. coli RecQ protein (29) are able to function as monomeric helicases in vitro. Yet with the exception of the Tra I helicase, the SF1 and SF2 helicases alone generally unwind DNA with low processivity in vitro. On the other hand, in a complex with accessory proteins, the processivity of some SF1 enzymes can be increased dramatically. For example, E. coli RecB in complex with RecC is a highly processive SF1 helicase.

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3 The abbreviations used are: ss, single-stranded; ds, double-stranded; NLLS, nonlinear least squares.
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(30, 31), the phage Φx174 gene A protein increases the helicase processivity of E. coli Rep (32–34), and the RepD protein is a processivity factor for PcrA (35, 36). Interestingly, removal of a subdomain (subdomain 2B) from the E. coli Rep protein activates the helicase activity of the monomer, indicating that the 2B subdomain is autoinhibitory for Rep monomer helicase activity (23). The hepatitis C NS3 SF2 enzyme also appears to require dimerization to unwind RNA in vitro (37). Hence, translocation and helicase activities for some SF1 and SF2 enzymes are separable in that the different activities require different oligomeric forms of the protein (7, 14, 22–26, 38–40).

_Bacillus stearothermophilus_ PcrA is an SF1 helicase with a monomeric structure (41) that is very similar to the monomer structures of both _E. coli_ Rep (42) and _E. coli_ UvrD (43). Based on structural and biochemical studies of the _B. stearothermophilus_ PcrA protein, it has been proposed that a PcrA monomer is the active form of the helicase in vitro (18), and a monomeric inchworm model has been proposed for how a monomer of PcrA might unwind DNA (18). Yet the suggestion that a monomer is the active form of the PcrA helicase in vitro is based on three pieces of circumstantial evidence. The first is that only a PcrA monomer is observed in crystal structures of PcrA bound to a 3′-ss/dsDNA junction (10-bp DNA duplex with a 7-nucleotide 3′ ssDNA flanking region) (44). The second is that a PcrA monomer possesses the ability to translocate processively with 3′ to 5′ directionality along ssDNA (5, 6). The third is that only PcrA monomers are observed in the absence of DNA when examined by gel filtration at high salt concentrations (200 mM NaCl) (45). Based on these results it has been assumed that a PcrA monomer must also be responsible for helicase activity in vitro. Yet definitive tests using single turnover kinetic approaches of whether _B. stearothermophilus_ PcrA monomers possess processive helicase activity in vitro have not been reported.

In this report we examine the ssDNA translocation and helicase activities of the _B. stearothermophilus_ PcrA monomer using single turnover kinetic approaches. These studies demonstrate that although PcrA monomers possess robust directionally (3′ to 5′) ssDNA translocation activity, in agreement with previous studies (5, 6), PcrA monomers are unable to unwind DNA duplexes as short as 18 base pairs. Hence, just like Rep and UvrD monomers, PcrA monomers are very good ssDNA translocases, yet are ineffective as helicases in vitro.

**EXPERIMENTAL PROCEDURES**

Buffers and Miscellaneous Stocks—Buffers were made with reagent grade chemicals using distilled water that was further deionized using a Milli-Q water purification system (Millipore Corp., Bedford, MA). Buffer A is 50 mM Tris (pH 7.5 at 25 °C), 1 mM diithothreitol, 2 mM EDTA. Storage Buffer is 50 mM Tris (pH 7.5 at 25 °C), 200 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, 2 mM 2-mercaptoethanol. Heparin buffer is 20 mM Tris (pH 7.5 at 25 °C), 10 mM NaCl, 1.5 mM MgCl₂, ssDNA buffer is 10 mM Tris-HCl (pH 7.5 at 25 °C) and 50 mM NaCl. Buffer H is 53 mM Tris (pH 7.5 at 25 °C), 151 mM NaCl, 3.2 mM MgCl₂, 1.4% (v/v) glycerol. Buffer L is 20 mM Tris (pH 7.5 at 25 °C), 10 mM NaCl, 3 mM MgCl₂, 10% (v/v) glycerol, 4 mM diithothreitol.

Buffer T is 20 mM Tris (pH 7.5 at 25 °C), 50 mM NaCl, 3 mM MgCl₂, 10% (v/v) glycerol, 4 mM diithothreitol. Spectrophotometric grade glycerol (Aldrich) was used for these buffers. TBE buffer is 89 mM Tris-base, 89 mM boric acid, 2 mM EDTA-Na₃ (pH 8.3 at 25 °C). ATP (Sigma-Aldrich) stock solutions were prepared in 50 mM NaOH (pH 7.5), and 500-µl aliquots were stored at −20 °C. ATP concentrations were determined spectrophotometrically using an extinction coefficient (259 nm) of 15.4 × 10⁻³ M⁻¹ cm⁻¹. Heparin (sodium salt) (Sigma-Aldrich; catalog number H-3393) stocks were dialyzed versus Heparin buffer at 4 °C, and the concentrations were determined as described (46).

PcrA Purification—_B. stearothermophilus_ PcrA was overexpressed in _E. coli_ BL21(DE3)pLysS cells, from plasmid pET-22b-pcrA, containing the wild type PcrA gene, kindly provided by P. Souljanas (University of Nottingham, Nottingham, UK) and purified from 2 liters of culture, essentially as described (45, 47, 48). All of the purification steps were performed at 4 °C with some modifications as follows. Protein eluted from the 40-ml Heparin Poros column (Applied Biosystems, Foster City, CA) was loaded onto a 40-ml High Q Poros column (Applied Biosystems) equilibrated with buffer A plus 0.1 M NaCl. The protein sample was diluted with buffer A as it was being loaded using the gradient mixer of the AKTA Purifier (GE Healthcare, Piscataway, NJ) such that the resulting conductivity of the diluted protein sample was no higher than that of the equilibration buffer. This was necessary to avoid precipitation of the PcrA. The High Q Poros column was washed with buffer A plus 0.1 M NaCl until the absorbance (280 nm) returned to baseline and the column was eluted with a 360-ml linear NaCl gradient (0.1–1 M NaCl) in buffer A (8 ml/min collecting 5-ml fractions). PcrA elutes at ∼180 mM NaCl. Fractions containing PcrA were pooled and loaded onto a ssDNA cellulose column (50 ml) equilibrated with buffer A plus 0.1 M NaCl by mixing directly with buffer A during loading as described above so that the conductivity was no higher than that of the equilibration buffer. The column was washed with buffer A plus 0.1 M NaCl and eluted with a 350-ml linear gradient (0.1–1 M NaCl) in buffer A (2.5 ml/min collecting 4-ml fractions). PcrA elutes at ∼700 mM NaCl. The peak fractions were pooled, and protein was precipitated by the addition of an equal volume of saturated ammonium sulfate in 50 mM Tris, pH 7.5 at 25 °C. The ammonium sulfate pellet was dissolved in 5 ml of buffer A plus 0.2 M NaCl, clarified by 30 min of centrifugation at 18,000 rpm, and applied (0.1 ml/min) to a 125-m1 Superdex 200 gel filtration column (GE Healthcare) equilibrated with buffer A plus 0.2 M NaCl. PcrA protein was dialyzed against Storage Buffer, flash frozen in liquid nitrogen, and stored at −80 °C. PcrA was determined to be >98% pure based on Coomassie Blue staining of serial dilutions run on an SDS-PAGE gel. PcrA concentration was determined spectrophotometrically in 6 M guanidinium hydroxycarhohide, 20 mM Tris (pH 7.5 at 25 °C) using ε₂₈₀ = 7.183 × 10⁴ M⁻¹ cm⁻¹ (0.87 ml mg⁻¹ cm⁻¹), calculated from its amino acid sequence (49, 50) using Sedenterp (51). The final yield was 0.5–1 mg of PcrA/g of _E. coli_ cell paste.

DNA—Unlabeled and Cy3-labeled oligodeoxynucleotides were synthesized and purified as described (52, 53) and dialyzed versus 10 mM Tris-HCl (pH 7.5) and stored at −20 °C. Oligode-
oxynucleotide concentrations were determined spectrophotometrically after digestion to a mixture of mononucleotides with snake venom phosphodiesterase I as described (7, 54). DNA unwinding substrates (3'−dT, 18bp) consisted of an 18-bp duplex region possessing a 3' ssDNA tail composed of \( n \) deoxythymidylates (dT\(_n\)). The sequence of the top strand (the strand without the 3' ssDNA tail) was 5'−GCCTCGCTGCGTCGCA−3'. Oligodeoxynucleotides were radiolabeled with \(^{32}\)P at the 5' end using T4 polynucleotide kinase (U.S. Biochemical Corp, Cleveland, OH), and purified as described (52). The duplex DNA was formed by annealing radiolabeled top strand (~600 nM) with 1.25-fold molar excess of unlabeled complementary bottom strand in ssDNA buffer by heating to 95 °C for 5 min and slow cooling to room temperature over a period of 2 h. Duplex DNA formation was confirmed by native PAGE on 10% acrylamide gel in TBE buffer. Poly(dT) (Midland Certified Reagents, Midland, TX) was fractionated using size exclusion chromatography to obtain a high molecular weight fraction (average length, 3.5−16 kb) as described (7) and was stored at −20 °C after dialysis versus ssDNA buffer.

**ssDNA Translocation Kinetics—PcrA monomer translocation** along ssDNA was examined in both Buffer L at 25 °C and Buffer H at 20 °C as indicated using an SX18MV stopped flow instrument (Applied Photophysics Ltd., Leatherhead, UK) under single turnover (single round) conditions (no rebinding of dissociated PcrA to ssDNA) by monitoring the arrival of PcrA at the 5' end of the DNA using 5'−Cy3-(dT)\(_n\), as described (7). PcrA was preincubated with ssDNA in the appropriate buffer (Buffer H or Buffer L) in one syringe, and the reactions were initiated by mixing in a 1:1 volume ratio with the same buffer containing ATP and heparin. All of the concentrations given are the final concentrations after mixing in the stopped flow. For experiments performed in Buffer L, the final solution conditions were: 40 nM PcrA monomer, 120 nM ssDNA, 2.5 mM ATP, 4 mg/ml heparin at 25 °C. For experiments performed in Buffer H, the final solution conditions were: 20 nM PcrA monomer, 100 nM ssDNA, 0.5 mM ATP, 4 mg/ml heparin at 20 °C. Control experiments show that when PcrA in one syringe is rapidly mixed with ssDNA and heparin in the other syringe, under identical solution conditions and concentrations, no translocation is observed, indicating that all of the free PcrA is trapped by the heparin.

Multiple time courses for PcrA translocation determined for a series of DNA lengths with 5'−Cy3-(dT)\(_n\), were analyzed globally by nonlinear least squares (NLLS) analysis using Equation 1 (schemes I and 2) to obtain estimates of the kinetic parameters as described (7).

\[
f(t) = \frac{A}{1 + nr} \left( \frac{1}{s + k_{\text{end}}} \right) \times \left( 1 + \frac{k_f}{s + k_d} \left( 1 - \left( \frac{s + k_l}{s + k_l + k_d} \right)^n \right) \right)^{-1}
\]  

(Eq. 1)

In Equation 1, \( \mathcal{L}^{-1} \) is the inverse Laplace transform operator; \( s \) is the Laplace variable; \( A \) is the signal associated with protein initially bound to ssDNA; \( k_l \) is the translocation rate constant; \( n \) is the maximum number of steps required for PcrA to translocate from the 3' end to the 5' end of a ssDNA of length \( L \), and taking \( m \) nucleotides per kinetic step, \( r \) is the ratio of the probability of PcrA binding to any position on the DNA other than the 5' end to the probability of protein binding to the 5' end. The rate constants, \( k_d \) and \( k_{\text{end}} \), are for PcrA dissociation from the internal sites and the 5' end of DNA, respectively. In this analysis, \( k_p \) and \( k_d \) were floated as global parameters (i.e. constrained to be the same for all ssDNA lengths), whereas \( k_l \) was constrained to the value determined independently by monitoring dissociation from poly(dT) as described (7). In the NLLS analysis, the parameters \( A \) and \( n \) were allowed to float for each ssDNA length. The uncertainties in the fitted parameters (68% confidence limits) were determined by performing a 100 cycle Monte Carlo (55) simulation routine in Conlin (56). The maximum number of translocation steps, \( n \), for a ssDNA of length, \( L \) (nucleotides), is related to \( m \) and the PcrA contact size, \( d \) (nucleotides) by Equation 2.

\[
n = \frac{L - d}{m}
\]  

(Eq. 2)

**Kinetics of PcrA Monomer Dissociation from poly(dT)—**The dissociation rate constant of PcrA monomer from internal ssDNA sites, \( k_{\text{diss}} \), during PcrA translocation was measured using poly(dT) by monitoring the enhancement of PcrA tryptophan fluorescence upon dissociation from the poly(dT) as described (7). The final solution conditions after mixing were 40 nM PcrA monomer, 2.5 mM ATP, 4 mg/ml heparin, 12 \( \mu \)M poly(dT) (in units of nucleotides) in Buffer L at 25 °C or 20 nM PcrA monomer, 0.5 mM ATP, 4 mg/ml heparin, 10 \( \mu \)M poly(dT) (in units of nucleotides) in Buffer H at 20 °C.

**Single Turnover DNA Unwinding Time Courses—**DNA unwinding experiments under single turnover conditions were performed using a three pulsed quenched flow apparatus (KinTek RQF-3, University Park, PA) as described (20, 24). PcrA was preincubated with the DNA substrate (cold DNA was spiked with 1 nM \(^{32}\)P-labeled DNA of the same sequence) on ice for 20 min in Buffer L or Buffer H and then loaded in one loop (18 \( \mu l \)) of the quenched flow apparatus. The other loop was loaded with Buffer L containing 2.5 mM ATP, 4 mg/ml heparin (a trap for free PcrA protein), and 2.5 \( \mu \)M of the unlabeled top strand of the DNA substrate (to prevent readannealing of the unwound DNA) or Buffer H containing 0.5 mM ATP, 4 mg/ml heparin, and 2.5 \( \mu \)M unlabeled top DNA strand. All of the concentrations are final concentrations after mixing. The two loops were incubated for 5 min at the indicated temperature before initiating the reaction by mixing. The reactions were terminated by mixing with quench solution (0.4 mM EDTA + 10% (v/v) glycerol) after time intervals ranging from 40 ms to 60 s. The background fraction of ssDNA at \( t = 0 \) was determined by mixing the PcrA-DNA complex with Buffer H or Buffer L containing traps but in the absence of ATP, and these values were subtracted from each time point. An aliquot of 10% (v/v) SDS solution was added to each sample to a final concentration of 1% and mixed by vortexing. The samples were analyzed by electrophoresis in a nondenaturing 10% polyacrylamide gel to separate the ssDNA product from the dsDNA substrate. The radioactivity in each band was quantified using a STORM 840...
phosphorimager (GE Healthcare), and the fraction of DNA duplexes unwound as a function of time, \( f(t) \), was calculated using Equation 3 (57),

\[
  f(t) = \frac{C_s(t) - C_{s,0}}{C_{s,0} + C_{D,0}} \left( 1 - \frac{C_{s,0}}{C_s(t) + C_D(t)} \right) \tag{Eq. 3}
\]

where \( C_s(t) \) and \( C_D(t) \) are the radioactive counts within each band corresponding to the ssDNA and duplex DNA, respectively, at time \( t \). \( C_{s,0} \) and \( C_{D,0} \) are the corresponding quantities at \( t = 0 \). Single turnover DNA unwinding time courses were analyzed using Equation 4 (scheme 3) as described (21, 24),

\[
  f_{ss}(t) = A_x \left[ 1 - \frac{\Gamma(n,k_{obs}t)}{\Gamma(n)} e^{-\frac{x}{k_x}} (1 - x) \right] \times \left( \frac{k_{obs}}{k_{obs} - k_{hp}} \right)^n \left[ 1 - \frac{\Gamma(n(k_{obs} - k_{hp})t)}{\Gamma(n)} \right] \tag{Eq. 4}
\]

where \( f_{ss}(t) \) is the fraction of DNA molecules fully unwound at time \( t \), \( A_x \) is the total unwinding amplitude, \( x \) is the fraction of DNA molecules with productively bound PcrA, \( n \) is the number of steps required to unwind a duplex of length \( x \), \( k_{obs} = k_x + k_{hp} \), where \( k_x \) is the unwinding rate constant, \( k_{hp} \) is the dissociation rate constant at each step, and \( k_{hp} \) is the rate constant limiting the isomerization from nonproductive to productive PcrA-DNA complexes, \( \Gamma(n,k_{obs}t) \) is the gamma function of the variable \( n \), \( \Gamma(n,k_{obs}t) \) is the incomplete gamma function of \( n \) and \( k_{obs}t \), and \( n = L/m \), where \( m \) is the kinetic step size (base pairs of DNA unwound per repeated rate-limiting step).

Single Time Point Single Turnover DNA Unwinding—Single time point DNA unwinding experiments were performed as follows. PcrA (at the indicated concentration) was preincubated with a DNA substrate (25 nM) in Buffer L (50 \( \mu \)l) for 20 min on ice followed by 5 min at 25 °C. The reactions were initiated at 25 °C by mixing the PcrA-DNA solution with 50 \( \mu \)l of Buffer containing 2.5 mM ATP, 4 mg/ml heparin (a protein trap), and 2.5 \( \mu \)l of unlabelled top strand of the DNA substrate to prevent renaturation of the DNA. The reactions were quenched after 130 s by addition of 100 \( \mu \)l of quench solution. The extent of DNA unwinding was quantified as described above for the quenched flow experiments.

Multiple Turnover DNA Unwinding—Multiple turnover DNA unwinding experiments were performed as follows. DNA substrate (3′-dT20-18bp) (1 nM) was preincubated with 2.5 mM ATP for 5 min at 37 °C in 50 \( \mu \)l of Buffer T. DNA unwinding was initiated by the addition of PcrA (varying concentrations) in 50 \( \mu \)l of Buffer T (preincubated at 37 °C for 5 min). The reaction was allowed to proceed for 130 s at 37 °C, after which it was quenched by mixing with 100 \( \mu \)l of quench solution containing 50 \( \mu \)M DNA trap (unlabeled top strand of the DNA substrate). The products of the reaction were analyzed and quantified as described above.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay (EMSA) experiments were performed in Buffer L at 25 °C. 50 nM of DNA substrate possessing a 18-bp duplex region and 3′ single-stranded tail (dT7 or dT20) was incubated with increasing concentrations of PcrA (10–400 nM) for 20 min at 4 °C followed by 5 min at 25 °C. 10 \( \mu \)l of samples were loaded onto 5% native polyacrylamide gel prepared in 0.25× TBE buffer to separate the free DNA from PcrA-DNA complexes. Electrophoresis was conducted at 25 °C in 0.25× TBE buffer at 200 V for 45–60 min.

RESULTS

PcrA Monomer Translocation along ssDNA—It was shown previously that monomers of *B. steatorrhophilus* PcrA are able to translocate with biased 3′ to 5′ directionality along ssDNA (5, 6). Hence we first examined the ssDNA translocase activity of our preparation of PcrA to compare with these previous results. We used a stopped flow fluorescent assay introduced by Dillingham *et al.* (5) with modifications as described (7, 58) to ensure that only a single round of translocation occurs (single turnover with respect to translocation, not ATP hydrolysis). Because the PcrA monomer is a 3′ to 5′ ssDNA translocase (5), we used a series of oligodeoxythymidylicates ((dT)\(_L\)), varying in length \( L \), that have a fluorophore (Cy3) attached covalently to their 5′ end. Upon reaching the 5′ end of the ssDNA, the PcrA translocase interacts with the Cy3 fluorophore, resulting in an enhancement of Cy3 fluorescence intensity. The experiment is performed by preincubating PcrA with the 5′-Cy3-(dT)\(_L\), under conditions of excess DNA and starting the reaction by the addition of buffer containing ATP and heparin. The heparin is included to serve as a trap for free PcrA protein, thus preventing rebinding of PcrA that dissociates during the course of translocation. Thus each monomer is restricted to one round of translocation, which simplifies analysis of the kinetic time courses. The kinetic parameters (rate and processivity) describing ssDNA translocation can be obtained from quantitative analysis of the time courses obtained for several experiments performed as a function of ssDNA length, \( L \) (7, 58).

We performed ssDNA translocation experiments under two sets of solution conditions (Buffer H and Buffer L) that differ in the [NaCl] (151 versus 10 mM), glycerol concentration (1.4% versus 10% (v/v)), and temperature (20 °C versus 25 °C). These two conditions were also used in the DNA unwinding experiments discussed below. We first performed experiments in Buffer H (53 mM Tris, pH 7.5, 151 mM NaCl, 3.2 mM MgCl\(_2\), 1.4% glycerol) at 20 °C, conditions similar to those used in previous studies of PcrA monomer translocation (5). PcrA protein (20 nM) was incubated with 5′-Cy3-(dT)\(_L\) (\( L = 64, 79, 97, 101, 104, 114, \) or 124 nucleotides) (100 nM) (all concentrations indicated are the final concentrations after mixing in the stopped flow). The reactions were initiated by mixing Buffer H containing ATP (500 \( \mu \)M) and heparin (4 mg/ml). The resulting time courses (Fig. 1A) display an ATP-dependent enhancement of Cy3 fluorescence reflecting arrival of translocating PcrA monomer at the 5′ end of the (dT)\(_L\), followed by a decrease in Cy3 fluorescence because of dissociation of the PcrA translocase from the 5′ end of the ssDNA. As the length of (dT)\(_L\) is increased, the position of the peak in the Cy3 fluorescence time course is shifted to longer times, reflecting the longer average time required to translocate along the longer ssDNA. These results indicate that PcrA monomers...
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FIGURE 1. ssDNA translocation kinetics of PcrA monomers under single turnover conditions. A, stopped flow kinetic experiments performed (20 °C, Buffer H) with PcrA (20 nM) and a series of ssDNA molecules (5'-Cy3-(dT)) with L = 64, 79, 97, 101, 104, 114, and 124 nucleotides (100 nM) while monitoring the increase in Cy3 fluorescence upon the arrival of PcrA at the 5' end of the ssDNA. The solid lines are time courses simulated using Equation 1 and the kinetic parameters (see Table 1) obtained from a global NLLS fit of all time courses. The reactions were initiated by rapid mixing with 0.5 mM ATP and 4 mg/ml heparin. B, the maximum number of steps, n, obtained from global NLLS analysis of the time courses in A, plotted as a function of ssDNA length, L, and fit to Equation 2. C, stopped flow kinetic experiments were performed as described above, but in Buffer L at 25 °C with 40 nM PcrA and 120 nM 5'-Cy3-(dT). The reactions were initiated by mixing with 2.5 mM ATP and 4 mg/ml heparin. D, the maximum number of steps, n, obtained from global NLLS analysis of the time courses in C, plotted as a function of ssDNA length, L, and fit to Equation 2.

can translocate efficiently and with biased 3' to 5' directionality along ssDNA in agreement with previous studies (5). Hence, the PcrA protein used in these experiments is a processive 3' to 5' ssDNA translocase.

The kinetic parameters describing the ssDNA translocation by PcrA monomers were obtained by analysis of the kinetic time courses using Equation 1, which is derived from the sequential "n step" mechanism shown in schemes 1 and 2 (Fig. 2) (7, 58). The model assumes that a PcrA monomer binds randomly but with polarity to a ssDNA, L nucleotides long with a contact size of d (nucleotides). The protein is assumed to bind initially with equal probability to any stretch of d nucleotides (i.e. potential end effects are ignored) using its full contact size even when bound to the ends of the DNA (i.e. no dangling protein). Upon initiation of ATP, the PcrA translocase moves with 3' to 5' directional bias and finite processivity along the DNA via a series of repeated translocation steps with rate constant, k. During each translocation step PcrA moves m nucleotides and can dissociate from any internal DNA site with internal ssDNA sites was constrained based on an independent determination made using poly(dT) by monitoring the increase in intrinsic tryptophan fluorescence of PcrA upon dissociation as described under "Experimental Procedures." In Buffer H, $k_d = (0.29 \pm 0.01) \text{ s}^{-1}$. Under these conditions, the PcrA monomer translocates with a kinetic step size, $m = (4.3 \pm 0.2)$ nucleotides (Fig. 1B) and $k_i = (19.8 \pm 0.7) \text{ s}^{-1}$. The macroscopic rate of translocation, $mk_r = (84.6 \pm 0.8)$ nucleotides s$^{-1}$, is similar to the rate (80 nucleotides s$^{-1}$) reported in previous studies of PcrA monomer translocation along oligodeoxythymidydrates labeled with 2-aminopurine (5) and slightly higher than the rate of translocation (50 nucleotides s$^{-1}$) determined from analysis of the time course of ATP hydrolysis during translocation (6). Under these conditions (Buffer H, 4 mg/ml heparin), the average translocation processivity, $P = mk_r/(mk_r + k_d) = 297 \pm 3$ nucleotides, representing the average number of nucleotides translocated by each PcrA monomer before dissociation from the ssDNA. The average translocation processivity in the absence of heparin is higher

rate constant, $k_p$. When PcrA reaches the end of the DNA, it dissociates with rate constant, $k_{end}$. Inclusion of a protein trap (heparin) with the ATP prevents any free PcrA that dissociates during translocation from rebinding ssDNA. We note that the "translocation rate constant," $k_p$, represents the rate constant for the slowest step that occurs within each repeated translocation cycle and does not necessarily correspond to the rate constant for movement of PcrA along the DNA (58). The average number of nucleotides translocated between two successive rate-limiting steps is defined as the translocation "kinetic step size," $m$. The PcrA monomer is assumed to bind initially at a random position along the ssDNA, i.e. translocation steps away from the 5' end, with concentration, $I_i$ (Fig. 2; scheme 2). The number of translocation steps, $i$, needed to reach the 5' end is constrained ($1 \leq i \leq n$), where $n$ is the maximum number of translocation steps needed for a PcrA monomer bound at the 3' end to move to the 5' end of a DNA. The concentration of PcrA bound at the 5' end is $I_{end}$ and free protein, $P$, is prevented from rebinding to DNA by the heparin.

NLLS analysis of the time courses in Fig. 1A yields the kinetic parameters summarized in Table 1. For this analysis, the rate constant for dissociation, $k_d$, of PcrA monomer from
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Table 1. ssDNA translocation kinetic parameters for PcrA monomer

| Translocation kinetic parameters | Buffer H* | Buffer L* |
|----------------------------------|-----------|-----------|
| \( k_i \) (kinetic steps s\(^{-1}\)) | 19.8 ± 0.7 | 19.8 ± 0.7 |
| \( k_{in} \) (s\(^{-1}\)) | 0.95 ± 0.02 | 0.95 ± 0.02 |
| \( k_{out} \) (s\(^{-1}\)) Cy3 | 12.7 ± 0.2 | 12.7 ± 0.2 |
| \( r \) | 6.6 ± 2.0 | 6.6 ± 2.0 |
| \( m \) (nucleotides step\(^{-1}\)) | 0.8 | 0.8 |
| \( d_{app} \) (nucleotides) | 0.4 ± 0.1 | 0.4 ± 0.1 |
| \( m_0 \) (nucleotides s\(^{-1}\)) | 233.7 ± 1.7 | 233.7 ± 1.7 |
| \( 1/(1 - P) \) (nucleotides) | 248 ± 2 | 248 ± 2 |

* Buffer H is 53 mM Tris, 151 mM NaCl, 3.2 mM MgCl\(_2\), 1.4% glycerol, pH 7.5. The experiment was performed at 20 °C.

* Buffer L is 20 mM Tris, 10 mM NaCl, 3 mM MgCl\(_2\), 10% glycerol, 4 mM dithiothreitol, pH 7.5. The experiment was performed at 25 °C.

* Measured independently in the dissociation assay using poly(dT). This parameter was constrained in the NLLS analysis of translocation kinetic.

* The higher than expected values of \( d_{app} \) result from analyzing data only from Cy3-labeled ssDNA, as we observed this same result with UvrD (7).

* Macroscopic translocation rate constant.

(339 ± 3 nucleotides) because heparin can actively displace PcrA monomers during translocation.

We also performed ssDNA translocation experiments in Buffer L, which contains 10 mM NaCl (20 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl\(_2\), 10% (v/v) glycerol, 4 mM dithiothreitol) at a higher temperature of 25 °C (Fig. 1, C and D). NLLS analysis of these time courses yielded the kinetic parameters listed in Table 1. Under these conditions, PcrA monomers also showed processive translocation along ssDNA, although with a ~2.6-fold higher macroscopic translocation rate of \( m k_i = (233.7 ± 1.7) \) nucleotides s\(^{-1}\) but a similar kinetic step size \((m = 4.0 ± 0.1\) nucleotides) and processivity \((P = 248 ± 2\) nucleotides). We note that the apparent value of the protein contact size, \( d_{app} \), obtained from these studies is higher than expected (~13 and 16 nucleotides/monomer). This appears to be the result of analyzing translocation data obtained only from Cy3-labeled ssDNA. We also observed higher than expected values of \( d_{app} \) upon analysis of UvrD monomer translocation data obtained only with Cy3-labeled ssDNA (7). When UvrD data obtained with fluorescein was also analyzed in combination with the Cy3 data, lower values of \( d_{app} \) (8–10 nucleotides) were obtained (7). Hence, the higher values of \( d_{app} \) obtained from analyzing data for ssDNA labeled only with Cy3 appear to reflect a fluorophore-dependent phenomenon, which is why analysis of data obtained from ssDNA labeled with different fluorophores yields more accurate estimates of \( d_{app} \). As discussed previously (7), the values of the translocation kinetic parameters are unaffected by the fluorophore.

DNA Helicase Activity of PcrA under Multiple Turnover Conditions—We next examined the helicase activity of PcrA under multiple turnover conditions to compare our results with those from previous studies (9). For these experiments we used an 18-bp duplex DNA possessing a 3'-dT\(_{20-18bp}\) tail (3'-dT\(_{20-18bp}\) and examined DNA unwinding as a function of [PcrA]. Unwinding was initiated by the addition of PcrA in Buffer T (20 mM Tris, 50 mM NaCl, 10% (v/v) glycerol, 4 mM dithiothreitol, 3 mM MgCl\(_2\), pH 7.5) at 37 °C to a solution of DNA (1 nM) and ATP (2.5 mM) in Buffer T as described (9). At 130 s the reaction was quenched with an equal volume of quench solution (0.4 M EDTA, pH 8.0, and 10% (v/v) glycerol). Fig. 3 shows the fraction of DNA unwound as a function of [PcrA]. These results demonstrate that under these multiple turnover conditions ~95% of the DNA duplexes (1 nM) can be unwound in the presence of excess PcrA (200 nM). This is similar to the helicase activity reported previously (9).

PcrA Monomers Show No Detectable Helicase Activity under Single Turnover Conditions—One cannot conclude from multiple turnover DNA unwinding experiments whether monomers of PcrA are active in DNA unwinding. We therefore used single turnover DNA unwinding experiments to assess whether a PcrA monomer is able to unwind duplex DNA (3'-dT\(_{20-18bp}\)) in vitro, as we have done previously with UvrD (20, 24, 26) and Rep (22, 23). In these single turnover experiments monomeric
PcrA protein was prebound to the DNA substrate, and a trap for free PcrA protein (4 mg/ml heparin) and a ssDNA complementary to one of the strands of the duplex DNA (to prevent reannealing of DNA) were added with the ATP to start the reaction. As a result, any DNA unwinding must be due to the activity of a PcrA monomer because additional PcrA is prevented from binding the DNA by the presence of the protein trap.

DNA unwinding was examined under the same two sets of solution conditions (Buffer H (20 °C) and Buffer L (25 °C)) that we used to examine PcrA monomer translocation along ssDNA. Both experiments were performed using [PcrA] and [DNA] identical to those used in the ssDNA translocation experiments with DNA substrate in molar excess over PcrA. In Buffer H, 20 nM PcrA was preincubated with 100 nM DNA, and reactions were initiated by the addition of 0.5 mM ATP and protein and DNA traps, whereas in Buffer L, 40 nM PcrA was preincubated with 120 nM DNA and rapidly mixed with 2.5 mM ATP and both traps. Sedimentation equilibrium experiments (supplemental Fig. S1) showed that no more than one PcrA monomer was bound per DNA substrate under these conditions of a molar excess of DNA.

As shown in Fig. 4, no DNA unwinding by PcrA monomers was detectable under single turnover conditions under either set of solution conditions. Yet importantly, under both sets of solution conditions, PcrA monomers can translocate along ssDNA with rapid rates and processivities far exceeding the length of the duplex DNA (18 bp) used in the unwinding experiments (Fig. 1 and Table 1). These results indicate that a PcrA monomer is unable to unwind even a short 18-bp DNA duplex, although PcrA monomers can translocate rapidly and processively along ssDNA under the same solution conditions.

Single turnover DNA unwinding experiments were also performed in the presence of a molar excess of PcrA protein (300 nM) over DNA substrate (25 nM). Two 18-bp duplex DNA substrates were used that differ by the lengths of the 3’ single-stranded oligodeoxynucleotide tails. In one set of experiments, an 18-bp duplex DNA with a seven nucleotide 3’-(dT)7 tail (substrate 3’-dT7-18bp) was used because this tail length should be short enough to allow binding of only one PcrA monomer (44). In the second set of experiments, an 18-bp duplex with a 3’-(dT)20 tail (substrate 3’-dT20-18bp) was used because this can bind at least two monomers of PcrA. The experiments were performed at 25 °C in Buffer L. 300 nM PcrA was preincubated with 25 nM DNA, and unwinding was initiated by rapid mixing with 2.5 mM ATP and a trap for free PcrA (4 mg/ml heparin) to ensure single turnover unwinding conditions and a DNA trap (2 μM of ssDNA that is complementary to the bottom strand of the DNA substrate). Rapid chemical quenched flow experiments were performed, and the resulting time courses were analyzed according to scheme 3 (Fig. 2) using Equation 4 as described (24).

As shown in Fig. 5 (A and B), no detectable unwinding was observed for the 18-bp duplex possessing only a 3’-(dT)7 tail under these single turnover conditions even at a 12-fold molar excess of PcrA to DNA. On the other hand, duplex DNA with a 3’-(dT)20 single-stranded tail could be unwound under conditions of a molar excess of PcrA, although with an amplitude of only ~10%. These results further demonstrate that a PcrA monomer is unable to unwind even a short duplex (18 bp) in vitro.

As an additional test to determine whether PcrA is able to bind efficiently to the DNA unwinding substrates, we examined binding using an EMSA assay under the buffer and temperature conditions used in the unwinding experiments described above. The results (Fig. 5, C and D) show that at the 300 nM PcrA concentrations used in the DNA unwinding experiments PcrA is able to saturate the DNA substrates containing either a 3’-(dT)7, and a 3’-(dT)20 tail. Hence, the lack of detectable unwinding of the 3’-dT7-18bp DNA substrate is not due to a lack of binding of PcrA.

We also examined unwinding by PcrA monomers of an 18-bp duplex with a flanking 3’-(dT)20 tail that is adjacent to a
second duplex but separated by a nick. We examined this DNA substrate because it was suggested that the additional duplex beyond the nick might be needed to provide points of contact for the 2B domain of a monomer of UvrD (43). In fact, neither PcrA monomers (supplemental Fig. S2) nor UvrD monomers (not shown) show detectable helicase activity on such a DNA substrate.

The unwinding time course of the 3′-dT20-18bp DNA substrate shown in Fig. 5 (A and B) was analyzed using Equation 4. Because we did not examine unwinding as a function of duplex DNA length, we are unable to obtain separate estimates for the kinetic step size, $m$, and $k_{obs} = (k_U + k_d)$ but can only estimate their product, the macroscopic unwinding rate, $mk_{obs} = (29 \pm 2)$ bp s$^{-1}$ (21, 59).

**DISCUSSION**

Previous suggestions that a *B. stearothermophilus* PcrA monomer is able to catalyze processive DNA unwinding *in vitro* have been based on three observations. First, only a monomer is observed bound to a 3′-ss/dsDNA junction with a 7-nucleotide flanking ssDNA in several crystal structures (44). Second, PcrA alone does not form stable dimers at high salt concentrations in *vitro* (45). Third, PcrA monomers are able to translocate with 3′ to 5′ directionality along ssDNA in an ATP-dependent reaction (5, 6). Although these observations are suggestive, no direct test had been reported for whether a PcrA monomer is able to catalyze DNA unwinding *in vitro*. We have now performed such a direct test using single turnover DNA unwinding experiments conducted under conditions such that no more than one molecule of PcrA is bound per DNA substrate. These experiments demonstrate that although the PcrA monomer is a rapid and processive ssDNA translocase, the monomer shows no detectable helicase activity in single turnover experiments even with a short 18-bp duplex DNA.

Interestingly, the behavior of the PcrA monomer *in vitro* is very similar to that observed for the *E. coli* Rep (14, 22, 23, 40) and *E. coli* UvrD proteins (7, 24–26), all of which are in the SF1superfamily of helicases. Although the studies reported here cannot rule out that a monomer can catalyze partial unwinding of one or a few base pairs of DNA, it is clear that DNA unwinding by a PcrA monomer is not sufficiently processive to unwind a duplex DNA even as short as 18 bp. Significant DNA unwinding *in vitro* is only observed when multiple PcrA monomers, which may interact functionally, are bound to the DNA substrate. At a minimum, our results indicate that the ability of these monomers to translocate processively and with directionality along ssDNA, although necessary, is not sufficient to confer processive helicase activity on these enzymes, and thus the translocation and helicase activities of these enzymes are clearly separable. As such, monomers of these enzymes should be referred to as translocases, rather than helicases *in vitro* because some additional activation (self assembly or interactions with accessory proteins) is required in order for them to become helicases.

PcrA (41, 44), Rep (42), and UvrD (43) all have very similar monomeric structures consisting of two domains (domains 1 and 2) that are further divided into four subdomains (1A, 2A, 1B, and 2B). The nucleotide-binding site is at the cleft between the 1A and 2A subdomains, whereas the ssDNA-binding site spans the “top” of the 1A and 2A subdomains. The 2B subdomain of each of these monomers is of similar size and can undergo significant rotation about a hinge region emerging from the 2A subdomain. In fact, the 2B subdomain within a Rep monomer can rotate by at least 130 degrees to form “open” and “closed” conformations (14, 42, 60). Crystal structures have been solved for both PcrA (44) and UvrD (43) monomers in complex with 3′-ss/dsDNA junctions. In these complexes, the 2B subdomain of both monomers is observed to be in a “closed” form conformation with the 2B subdomains in contact with the duplex DNA. Based on this, it was suggested that these structures represent “snapshots” of intermediates that exist along the pathway for DNA unwinding and detailed models have been proposed for how monomers of PcrA (44) and UvrD (43) might unwind DNA processively.

An alternative interpretation is that these structures (43, 44) represent autoinhibited complexes that are not on the pathway for productive unwinding. This suggestion is supported by the fact that monomers of Rep (14, 22, 23, 40, 61), UvrD (7, 24), and PcrA (this study) are unable to unwind DNA processively *in vitro*. Furthermore, because removal of the 2B subdomain of Rep to form RepΔ2B, actually activates its latent monomeric
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helicase activity, this indicates that the 2B subdomain is autoinhibitory for Rep monomer helicase activity (23, 61). This may also be true for UvrD and PcrA monomers. In fact, expression of the same 2B deletion of UvrD (61) is lethal in E. coli, possibly because of activation of an unregulated helicase. Therefore, we suggest that the 2B subdomain interactions with the duplex that are observed in the crystal structures of PcrA (44) and UvrD (43) may inhibit rather than facilitate DNA unwinding by these monomers. This interpretation is further supported by the fact that some mutations made within residues in the 2B subdomain of UvrD that were designed to inhibit helicase activity based on the model derived from the crystal structure actually enhance helicase activity (43). Such mutations may actually serve to relieve the inhibitory interactions of the 2B subdomain. We have suggested that the self-assembly of Rep and UvrD that activates their helicase activity in vitro may facilitate a conformational reorientation of the 2B subdomain so that it no longer inhibits DNA unwinding (23). The same may be true for PcrA. Hence, current models for processive DNA unwinding by monomers of PcrA (18, 44) and UvrD (43), based on the structures of monomers of PcrA and UvrD bound to ss/dsDNA junctions, may need to be reassessed if these structures actually represent autoinhibited monomeric complexes rather than intermediates along the DNA unwinding pathway. Although we cannot rule out that the crystallization process somehow “captured” intermediate structures that resemble the conformations of the activated monomers, this possibility is not supported by the activities of some mutations made within the 2B subdomain of UvrD (43).

Activation of the latent helicase activities of Rep, UvrD, and PcrA can also occur through interactions with accessory proteins. For instance, MutL can facilitate UvrD helicase activity (62–65), whereas the phage M13 gene II protein (66) and the phage Φ×174 gene A protein (32, 33, 67) can interact with Rep to enhance its DNA unwinding processivity. Similarly, the plasmid replication protein, RepD, has been shown to enhance the DNA unwinding processivity of PcrA (35, 36). These protein-protein interactions may function partly by affecting the conformation of the 2B subdomain to activate the latent helicase. In this regard, it is interesting to note that the 2B subdomain of the E. coli RecB helicase/translocase interacts with the 2B subdomain of its processivity factor, RecC, to form the highly processive RecBC(D) helicase (68). Hence, this may be an example of a protein-protein interaction that relieves the inhibitory effect of the 2B subdomain of an SF1 helicase/translocase.

Although PcrA, Rep, and UvrD monomers do not display helicase activity in vitro, there is clear evidence, using the same single turnover DNA unwinding experiments as used here, that some SF1 monomers do possess helicase activity in vitro. The phage T4 Dda monomer shows limited helicase activity with low processivity in vitro (69, 70), the TraI monomer encoded by the E. coli F episome is a highly processive helicase (28), and the SF2 RecQ monomer also displays helicase activity in vitro (29). Why some monomers can function as helicas, whereas others function only as translocases remains an outstanding question. Interestingly, the structure of the RecQ monomer (71) shows that it does not possess a domain that is equivalent to the 2B subdomain of Rep, UvrD, or PcrA. As such, it has been suggested that the RecQ monomer may have helicase activity because it is missing the autoinhibitory 2B subdomain (29), similar to what is observed for the RepΔ2B monomeric helicase (23).

Most DNA helicases are known to function in multiple processes in vivo. For example, E. coli UvrD functions in methyl-directed mismatch repair (72), DNA excision repair (73), replication restart (10, 74, 75), and plasmid replication (76). UvrD also dismantles RecA protein filaments formed on ssDNA (11) and clears proteins from replication forks (10). In fact, mutations in UvrD show hyper-recombinational phenotypes, presumably because of an inability to disrupt such filaments (12, 13, 77, 78). Although it has generally been assumed that the helicase activity of UvrD is the function that is required in all of these processes, it is possible that some of the UvrD functions require only its ssDNA translocase activity. In fact, ssDNA translocation may be the important activity needed for UvrD to displace RecA protein from DNA. This may also be true for Rep and PcrA. If so, then it might be advantageous to enable the translocase and helicase activities of these enzymes to function independently and be regulated. For example, if the monomeric UvrD ssDNA translocase were also able to function as a helicase without any need for activation, then after displacing RecA from a ssDNA gap, UvrD could proceed past the gap to unwind duplex DNA unnecessarily. The absence of helicase activity within the full-length monomeric Rep, UvrD, and PcrA translocases could serve to prevent unregulated unwinding of DNA within the cell.

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