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Citation
Kulczyk, Arkadiusz W., Barak Akabayov, Seung-Joo Lee, Mihnea Bostina, Steven A. Berkowitz, and Charles C. Richardson. 2012. “An Interaction between DNA Polymerase and Helicase Is Essential for the High Processivity of the Bacteriophage T7 Replisome.” Journal of Biological Chemistry 287 (46): 39050–60. https://doi.org/10.1074/jbc.m112.410647.

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An Interaction between DNA Polymerase and Helicase Is Essential for the High Processivity of the Bacteriophage T7 Replisome

Arkadiusz W. Kulczyk, Barak Akabayov, Seung-Joo Lee, Miheea Bostina, Steven A. Berkowitz, and Charles C. Richardson

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard University Medical School, Boston, Massachusetts 02115, the Facility for Electron Microscopy Research, McGill University, Montreal, Quebec H3A 2B2, Canada, and Biogen Idec, Inc., Cambridge, Massachusetts 02142

Background: Interactions of DNA polymerase and DNA helicase are crucial in DNA synthesis.

Results: Two distinct interactions are involved in formation of the DNA polymerase/DNA helicase complex.

Conclusion: The multiple interactions between DNA polymerase and DNA helicase account for the high processivity of leading strand synthesis.

Significance: Understanding of the replication process in bacteriophage T7 facilitates studies in more complex systems.

Synthesis of the leading DNA strand requires the coordinated activity of DNA polymerase and DNA helicase, whereas synthesis of the lagging strand involves interactions of these proteins with DNA primase. We present the first structural model of a bacteriophage T7 DNA helicase-DNA polymerase complex using a combination of small angle x-ray scattering, single-molecule, and biochemical methods. We propose that the protein-protein interface stabilizing the leading strand synthesis involves two distinct interactions: a stable binding of the helicase to the palm domain of the polymerase and an electrostatic binding of the carboxyl-terminal tail of the helicase to a basic patch on the polymerase. DNA primase facilitates binding of DNA helicase to ssDNA and contributes to formation of the DNA helicase-DNA polymerase complex by stabilizing DNA helicase.

Because of its simplicity, bacteriophage T7 provides a model system for the molecular interactions during DNA replication (1). Only four proteins are required for the basic phage replisome, yet the reconstituted replisome recapitulates all the key features of more complex systems (see Fig. 1A). The four proteins are: DNA polymerase and its processivity factor, Escherichia coli thioredoxin, DNA primase-helicase, and ssDNA-binding protein. The limited number of proteins has made possible: (i) the determination of their crystal structures, (ii) reconstitution of a replisome that mediates coordinated DNA synthesis, and (iii) visualization of active replisomes by single-molecule techniques.

The protein product of T7 gene 4 (gp4) provides helicase and primase activities. The helicase resides within the C-terminal half of the protein and the primase in the N-terminal half (2).

The primase contains the N-terminal zinc-binding domain (ZBD)2 connected to the RNA polymerase domain (RPD). The helicase domain uses the energy derived from the hydrolysis of dTTP to translocate unidirectionally on ssDNA (3). Upon encountering dsDNA, it unwinds the DNA. The primase domain recognizes specific sequences on the lagging strand and synthesizes tetraribonucleotides that are used as primers for the lagging strand DNA polymerase (4). T7 DNA polymerase (gp5) contains an N-terminal proofreading exonuclease domain and a C-terminal polymerase domain. gp5 forms a one to one complex with its processivity factor thioredoxin (trx) (5).

The crystal structure of the gp5 and trx complex (gp5/trx) bound to a primer/template revealed that trx binds to a 76-amino acid loop inserted into the thumb subdomain (thioredoxin-binding domain) (6).

Coordination of leading and lagging strand DNA synthesis, the formation and resolution of a replication loop, and the exchange of DNA polymerases implies that the four proteins interact with one another. A number of these interactions have been described (see Fig. 1A). The binding of trx to gp5 alters the configuration of the thioredoxin-binding domain such that it can clamp the DNA within the DNA-binding crevice (7). This interaction also remodels the thioredoxin-binding domain to create two solvent-exposed basic loops (exchange patch) to which gp4 and gp2.5 can bind (8). Interactions of the C-terminal tail of gp4 with the exchange patch of gp5 increase the processivity of the replisome from 5 to >17 kb by capturing gp5/trx that transiently dissociates (9, 10). Another electrostatic interaction between the 17 C-terminal amino acid tail of gp4 and a basic patch of gp5 (loading patch) is essential for the loading of gp5/trx onto the replication fork (11).

There is a third unidentified interaction that does not involve the C-terminal tail of gp4. gp5/trx has a processivity of only 0.8

2 The abbreviations used are: ZBD, zinc-binding domain; RPD, RNA polymerase domain; SAXS, small angle x-ray scattering; trx, thioredoxin; AUC, analytical ultracentrifugation; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; LMW, low molecular weight; Ru, resonance unit.

Received for publication, August 15, 2012, and in revised form, September 12, 2012. Published, JBC Papers in Press, September 13, 2012. DOI 10.1074/jbc.M112.410647

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 287 • NUMBER 46 • NOVEMBER 9, 2012

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 46, pp. 39050 –39060, November 9, 2012
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kb on ssDNA but a processivity of >17 kb during leading strand synthesis when coupled to gp4 (12). A deletion of the C-terminal tail of gp4 reduces the processivity to 5 kb. Therefore, a third, uncharacterized interaction of gp4 and gp5/trx is essential for the high processivity of 5 kb observed during DNA synthesis in the absence of gp5/trx exchange. In the current study, we dissect the interactions that stabilize the complex of gp4 and gp5/trx during leading strand synthesis.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Biochemical Assays**—Proteins were overexpressed and purified as described previously: WT gp4 and gp4ΔZBD (13), gp4 ΔC (14), gp4Δhelicase, gp4ΔCΔprimase, gp5, and trx (15). In the SPR, EM, and small angle x-ray scattering (SAXS) experiments, we use a gp5/trx in which two amino acids (Asp-5 and Glu-7) in the exonuclease domain of gp5 are replaced with Ala; the 3’ to 5’ proofreading exonuclease activity is reduced by a factor of 10^6 (6). The biochemical assays have been described in detail earlier: DNA binding and oligomerization assays (16), unwinding assay (17), and minicircle assay (18).

**Single-molecule Measurements**—Phage λ DNA (48.5 kb) molecules containing a replication fork were attached with the 5’-end of one strand to the glass surface of a flow cell via biotin-streptavidin link. The 3’-end of the same strand was attached to a 2.8-μm paramagnetic bead (Dynal) via digoxigenin-anti-digoxigenin. To prevent nonspecific interactions between the beads and the surface, a 1-pN magnetic force was applied upward by positioning a permanent magnet above the flow cell. The beads were imaged with a CCD camera with a time resolution of 500 ms, and the centers of their positions for every acquisition time point were determined by particle-tracking software. Bead-bound and surface-tethered DNA molecules were preincubated with 300 nM of different variants of gp4 (monomeric concentration), 100 nM gp5/trx in buffer: 40 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 2 mM EDTA, 0.1 mg/ml BSA, 10 mM DTT, consisting of 600 μM each of dATP, dTTP, dCTP, and dGTP. Next, the flow cell was washed with replication buffer with dNTPs. DNA synthesis was initiated by introducing replication buffer with dNTPs and 10 mM MgCl₂. For data analysis, after particle tracking, the traces were corrected for residual instabilities in the flow by subtracting traces corresponding to tethers that were not enzymatically altered. Bead displacements were converted into numbers of nucleotides synthesized using the known length difference between ssDNA and dsDNA at our experimental conditions (12).

**Surface Plasmon Resonance**—SPR analysis was performed using a Biacore 3000 instrument (Uppsala, Sweden). Binding experiments between gp5/trx and gp4 variants in the presence of primer/template were performed as previously described (8). The template 5’-CCCCTTGGCACGTGGCGTGTTTTCACGTT-biotin-3’ and the primer 5’-CGTTAAACGACGAGCGGCGTCGTTTCCGTGCAA-3’ strands were annealed, and 350 RU of a primer/template were coupled to the SA chip. As a control, one flow cell on the chip was blocked with free biotin. Binding studies were carried out in buffer containing 20 mM HEPES, pH 7.5, 200 mM potassium glutamate, 10 mM MgCl₂, 5 mM DTT, 1% (w/v) glycerol, and 0.5 mM GTP at a flow rate of 10 μl/min. gp5/trx was injected at the concentration of 200 nM in buffer containing 1 mM dGTP and 10 μM ddATP. gp4 variants were injected in buffer consisting of 0.1 mM ATP and 2 mM GTP. The flow cell blocked with biotin was used to measure the non-specific interactions and bulk refractive index. The data were analyzed using the software BIAEVAL 4.1.

**Sedimentation Velocity Analytical Ultracentrifugation**—AUC analysis of proteins was conducted using Beckman Coulter ProteomeLab XL-I centrifuge. The experiments were conducted at 20 °C using an eight-hole (An-50 Ti) rotor. Prior to AUC analysis, the samples were buffer-exchanged and concentrated using centrifugation cartridges with 10,000 molecular mass cutoff membranes into buffer consisting of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM MgCl₂, and 5 mM dTTP. The samples were then diluted to their target concentration using the same buffer used in conduct buffer exchange. All boundary sedimentation velocity runs were conducted at rotor speed of 40,000 or 45,000 rpm. AUC cells used for these experiments were assembled with 12-mm synthetic boundary double sector charcoal-filled epoC centerpieces and sapphire windows. The reference side of the double sector AUC cell was always filled with an excess of 20 μl of buffer solution in comparison with the samples sector. AUC cells were then placed into the rotor and accelerated to 15,000–20,000 rpm to transfer the excess buffer in the reference sector over to the samples sector, so the radial distance of the meniscus from the center of rotation for both the sample and reference sectors matched. Once buffer was transferred, the rotor was stopped, and the cells were removed and gently mixed by rotating the cell a number of times. The rotor, containing the cells, was then placed back into the centrifuge where it was allowed to temperature equilibrate for 1.5–2 h before the run was started. Data acquisition was carried out every 5 min using the Raleigh interferometer. Data analysis was conducted using the c(s) method in Peter Shuck’s program SEDFIT (19) using a regularization confidence level of 0.68 and data radial spacing of 0.003 cm.

**Electron Microscopy and Image Processing**—Uranyl formate samples were prepared as described (20). Images were collected at a Tecnai F-20 electron microscope (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 200 kV with a magnification of 50,000× corresponding to 2.25 pixel size, at a defocus in range of 0.5–1.4 μm. Particle images were selected for each sample using the software Signature (21). A stack of ∼5000 images were windowed into 128×pixel boxes. Images analysis was done using SPARX (22). Several rounds of K-means classification and multireference were applied.

**Small Angle X-ray Scattering**—The sample for SAXS measurements was prepared by mixing 30 μM gp4Δprimase bound to a 15-mer oligonucleotide (GGGGTCACGATCGTGATGACG) in the presence of β,γ-methylene dTTP with 5 μM gp5/trx bound to the previously annealed and purified 5.2 μM primer/template DNA (5’-CGAAAAACGACGAGCGAATGGCAG-3’/5’-CCCCCTTGGCGCCTGG-3’) in the presence of 0.1 mM ddATP, and 1 mM dGTP in the following buffer: 50 mM potassium phosphate, pH 7.5, 10% glycerol, and 1 mM DTT. The measurements were performed at the National Synchrotron Light Source (Upton, NY) on Beamline X-9. The magnitude of the scattering vector (q) is defined as follows,
where $\theta$ is the scattering angle.

The experimental SAXS data for the sample showed a linear behavior in the low $q$, Guinier region, indicating that the leading strand complex did not undergo aggregation. Radii of gyration ($R_g$) were derived from data in the $qR_g < 1.3$ region using the Guinier approximation.

$$I(q) = I(0)\exp\left(-\frac{R_g^2q^2}{3}\right)$$  \hspace{1cm} (Eq. 2)

The scattering curve reflects structural characteristics in reciprocal space. The software GNOM was used to translate the scattering profiles into real space by Fourier transformation, resulting in the pairwise-distance distribution function ($p(r)$). The $p(r)$ function is proportional to the particle electron density and reflects the distances between pairs of scattering points within the macromolecule, permitting determination of the maximum intramolecular diameter ($D_{max}$). To obtain a reliable quantification of the maximum diameter of the examined particles ($D_{max}$), we utilized the methods described earlier (23), which was incorporated into our in-house scripts. In this method, $D_{max}$ was set as a variable in the range of 100–300 Å and varied in increments of 10 Å. Evaluation parameters described by Lipfert et al. (23) were analyzed, and the values of $D_{max}$ that showed the best overall behavior and GNOM statistics were chosen for the subsequent structure reconstruction (supplemental Fig. S2).

Three-dimensional Structure Reconstruction—The three-dimensional shape of the gp4Δprimase-ssDNA in complex with gp5/trx-primer/template was obtained from the experimental scattering data using the software GASBOR (24). All rigid body analysis programs employ amplitudes from the computer program CRYSOL that also explicitly simulates the solvation shell by a 3 Å layer on the surface. GASBOR performs ab initio shape reconstruction that represents the molecular shape as a closely packed sphere assembly within a search volume defined by the maximum particle dimensions ($D_{max}$) chosen using the search script mentioned above. Ten low resolution models were averaged using the program DAMAVER to yield an averaged model representing the general structural features of each reconstruction.

RESULTS

Genetically Altered Variants of DNA Primase-Helicase—To determine the contributions of the individual domains of gp4 to interactions with gp5/trx, the WT gp4 was genetically altered to create five gp4 variants (Fig. 1B). gp4Δhelicase lacks the entire helicase domain. gp4ΔC lacks the 17 C-terminal amino acids. gp4ΔZBD lacks the N-terminal 63 residues, a deletion that removes the ZBD of the primase domain. gp4Δprimase is a more extensive deletion, resulting in removal of the entire primase domain. gp4ΔCprimase lacks the entire primase domain and the C-terminal amino acids. In the following section we assess the binding of these variants to gp5/trx.

Physical Interactions between the DNA Helicase Domain of gp4 and gp5/trx Stabilize the Leading Strand Complex—During DNA synthesis gp5/trx extends the leading strand, whereas the gp4 hexamer encircles the lagging ssDNA. Terminating the primer with a ddNMP and providing the next dNTP specified by the template yields a gp5/trx-primer/template complex that mimics ongoing leading strand synthesis, and its structure was solved to a 2.2 Å resolution (6). gp4 and gp5/trx form a stable complex under these conditions (8). Therefore, to dissect the interactions involved in formation of the leading strand replication complex, we have examined the ability of gp4 variants to bind to gp5/trx under these conditions using SPR. Fig. 2A shows a cartoon of gp5/trx locked in a polymerization mode on a primer/template.

The SPR sensorgrams in Fig. 2B show the binding between gp5/trx-primer/template and the different gp4 variants that were subsequently injected into the flow cell. Because the gp4 variants differ in mass from the WT gp4, we normalized the
data by multiplying the maximal RU ($R_{max}$) recorded for gp4 variants by the ratio of the mass of the WT protein to that of the altered protein (Fig. 2C).

WT gp4 forms a stable complex as evidenced by a rapid increase in RU after gp4 injection and a slow dissociation from the complex. gp4ΔZBD and gp4Δprimase bind at 112 ± 2 and 133 ± 13% of the level observed with WT gp4, respectively. Both of these gp4 variants associate with the complex in a similar manner as the WT protein. However, they dissociate at a faster rate. The data indicate that the ZBD and RPD contribute to the stability of the complex. The binding of gp4Δhelicase lacking the entire helicase domain but containing the primase domain supports this conclusion. gp4Δhelicase displays less than 10% of the level of binding measured for WT gp4. A similar stabilization effect is observed in the crystal structure of Bacillus stearothermophilus helicase bound to the helicase-binding domain of the primase (25).

As expected, a deletion of the C-terminal tail of gp4 (gp4ΔC) decreases the binding of gp4 (68 ± 9% of the level recorded for the WT protein) (9). To test directly whether DNA helicase is involved in formation of the leading strand complex, we measured binding of gp4ΔCΔprimase. gp4ΔCΔprimase binds at 31 ± 3% of the level observed with WT gp4. Thus the helicase domain of gp4 interacts with gp5/trx during leading strand synthesis. In a control, we found no detectable binding of gp4 to the immobilized DNA alone. The ZBD, RPD, and the helicase domain of gp4 are connected by flexible linkers. Therefore removal of these domains does not change the surface and thus does not create an artificial interaction with gp4.

Single-molecule Analysis of Leading Strand Synthesis—

Single-molecule techniques allow for the quantitative measurement of the rate and processivity of DNA synthesis catalyzed by individual replisomes (12, 26). Fig. 3A shows a schematic representation of the single-molecule assay we used. Linearized A DNA is modified to form a structure resembling the replication fork at one end. The fork-shaped DNA ligand has biotin on the 3'-end of one strand and a digoxigenin on the 5'-end of the same strand. The biotinylated end is attached to a coverslip, and the digoxigenin end is attached to a 2.8-μm bead. A laminar flow of 3 pN is applied, and the resultant drag stretches the molecule of A DNA. At 3 pN the elasticity of DNA is determined by entropic contributions, and ssDNA is shorter than dsDNA because of coiling of the ssDNA. Consequently, by measuring the decrease in length of the DNA molecule, we monitor conversion of dsDNA to ssDNA as a result of the leading strand synthesis. These measurements are accomplished by imaging the bead position as a function of time (Fig. 3A).

gp5/trx and gp4 were flowed over the immobilized DNA in buffer containing dNTPs. After loading onto the DNA, free protein was removed by washing the flow cell with buffer. Leading strand synthesis was initiated by flowing buffer containing MgCl$_2$. The processivity is determined by measuring the length of each DNA molecule from the beginning to the end of the shortening event. The rate is obtained by fitting each shortening trajectory with linear regression and calculating the slope. All individual processivity and rate measurements are then combined in distributions and fitted with exponential and Gaussian functions, respectively (Fig. 3B). Representative trajectories and the averaged values are shown in Fig. 3C.

With gp5/trx and WT gp4 synthesis proceeds at a rate of 112 ± 24 nucleotides/s and a processivity of 16 ± 4 kb. Substitution of WT gp4 with gp4ΔZBD results in a 27% decrease in rate and a 38% decrease in processivity, suggesting that the deletion of ZBD has a minimal influence on synthesis. Substitution of gp4ΔC for WT gp4 results in a 20% decrease in rate and a decrease in processivity to 4 kb. We showed previously that gp5/trx dissociates from the DNA approximately every 5 kb and can either be retained by the C-terminal tail of gp4 or exchange with free gp5/trx in solution (27). No shortening trajectories, indicative of no synthesis, were detected with gp4Δprimase and gp4ΔCΔprimase.

In the replication system of B. stearothermophilus, the interaction between the primase (DnaG) and the helicase (DnaB) increases the NTPase and the unwinding activities of DnaB (28) and consequently increases the processivity of replication. The crystal structure of DnaB bound to helicase-binding domain of DnaG shows that the conformation of DnaB hexamer is stabilized by binding of the three helicase-binding domain to the surface of DnaB (25). Analogically, the interaction between the primase and the helicase of gp4 fulfills this role during leading strand synthesis. The lack of activity exhibited by gp4Δprimase and gp4ΔCΔprimase can result from an inability of the trun-
cated proteins to oligomerize, weak ssDNA binding, or a deficiency in unwinding of dsDNA.

Analytical Ultracentrifugation Studies of the Oligomerization of gp4/H9004 primase—We used analytical ultracentrifugation to examine the oligomerization properties of gp4/H9004 primase and performed sedimentation velocity experiments (AUC) with interference optics. Three samples of gp4/H9004 primase were analyzed at different concentrations, in buffer containing MgCl₂ and dTTP. Fig. 4A shows the noise-corrected raw AUC data (upper panel) for the sample at 1 mg/ml and its associated calculated distributions of sedimentation coefficients (c(s)) plotted versus sedimentation coefficient (s) expressed in unit of Svedberg (S) (lower panel). The plot of c(s) versus s for each sample shows the presence of three groups of peaks: (i) <8 S corresponds to low molecular weight (LMW) oligomers, likely monomers, dimers, and trimers; (ii) 8–9 S, most likely the hexameric helicase; and (iii) >10 S corresponds to high molecular weight (HMW) aggregates.

The quantification of samples is presented in supplemental Table S1. The results show little change in species composition as a function of total protein concentration. Plotting the weight-average s (s_w) for each sample versus s_w reveals a decreasing relationship of s_w with increasing concentration (supplemental Fig. S1A). The data indicate the absence of any significant dynamic equilibrium behavior between any gp4/H9004 primase species present.

As a control we characterized WT gp4 at different concentrations. Fig. 4B shows the analogous data calculated for WT gp4 at 1.1 mg/ml. The c(s) plotted versus s for each gp4 sample shows the presence of two peaks between 11 and 14 S. Previous analysis of WT gp4 samples by electron microscopy revealed the presence of hexameric and heptameric rings with heptamers varying between 33% (29) and 78% (30). Therefore it is likely that these two peaks (the slow and fast migrating peaks) correspond to the hexameric and heptameric gp4, respectively. Although LMW and high molecular weight species are also...
The octameric ring is 10% larger than the heptameric ring, and gp4 primase and 15% for the WT gp4) display octameric rings. A significant fraction of particles (14% for primase and 37 and 48%, respectively, for gp4) revealed the presence of three different species (Fig. 4). Image classification revealed the presence of three different forms of gp4 in both samples: hexamers, heptamers, and octamers. The relative content of each species is specified in a table below the figure.

In WT gp4 samples, the percentage of LMW species is significantly lower than for gp4Δprimase (supplemental Table S2).

Supplemental Fig. S1B displays $s_w$ plotted as a function of the total protein concentration. In contrast to the data obtained for gp4Δprimase, the figure shows that $s_w$ increases with increasing concentration of WT gp4. Such concentration behavior is typical for a chemically reacting system. In summary, the results suggest the primase facilitates oligomerization of DNA helicase. The high percentages of LMW species detected for gp4Δprimase indicate that the primase stabilizes the ring of DNA helicase.

Analysis of gp4Δprimase Oligomerization by Electron Microscopy—We used negative staining EM to examine the oligomerization properties of gp4Δprimase and WT gp4. gp4 samples were incubated in the buffer previously used for sedimentation velocity AUC. A representative image of the negatively stained sample is shown in Fig. 4C. Image classification revealed the presence of three different species (Fig. 4D). Hexameric and heptameric rings are present in both samples (34 and 52%, respectively, for gp4Δprimase and 37 and 48%, respectively, for WT gp4). A significant fraction of particles (14% for gp4Δprimase and 15% for the WT gp4) display octameric rings. The octameric ring is 10% larger than the heptameric ring, and its central channel is 20% larger than the central channel of the heptamer. gp4Δprimase and WT gp4 display similar oligomerization patterns at low protein concentration.

gp4Δprimase Binding to ssDNA—The binding of gp4 to a 27-mer oligonucleotide was measured in the presence of the nonhydrolysable β,γ-methylene dTTP using a nitrocellulose DNA binding assay (Fig. 5A). WT gp4 binds the oligonucleotide with a $K_d$ of 161 ± 47 nM. In comparison, gp4Δprimase binds the ssDNA 3-fold less tightly ($K_d$ of 411 ± 53 nM) and binds 30% of the oligonucleotide at the highest protein concentration. The 3-fold poorer binding of gp4Δprimase to ssDNA is likely due to the fact that gp4Δprimase does not contain the RPD that alone can bind ssDNA (31). It was proposed earlier that the primase facilitates loading of gp4 onto ssDNA (32).

As shown in the previous sections, gp4 exists in multiple oligomeric forms, but the functional form is a hexamer. Only hexamers are found to bind to ssDNA (30). Oligomerization of WT gp4 in the presence of ssDNA was examined earlier by PAGE under native conditions (17). Two bands seen in the gels represent the hexameric and heptameric forms. In the presence of ssDNA and β,γ-methylene dTTP, the slower migrating band disappears, indicative of conversion from the heptameric to the hexameric ring of gp4 (30). In the current study, we assessed the effect of ssDNA on the oligomerization of gp4Δprimase. Differ-
DNAPolymerase-DNA Helicase Interaction

**Figure 5. Characterization of ssDNA binding, DNA unwinding, and strand displacement synthesis of gp4Δprimase.** A, binding to ssDNA was measured in a nitrocellulose DNA binding assay. The reactions contained different concentrations of gp4Δprimase or WT gp4, 5'-32P-labeled 27-mer oligonucleotide, and β,γ-methylene dTTP. The quantity of protein bound ssDNA and free ssDNA was measured in a PhosphorImager. The graph shows percentages of bound ssDNA as a function of increasing concentrations of gp4Δprimase (filled circles) and WT gp4 (open circles). B, oligomerization of gp4Δprimase upon binding to ssDNA was assessed by native gel electrophoresis. Three different protein concentrations were incubated in the presence or absence of a 27-mer oligonucleotide in buffer containing MgCl2 and β,γ-methylene dTTP and then cross-linked with glutaraldehyde. The products of the cross-linking reaction were analyzed on the native gel. C, DNA unwinding activity of gp4Δprimase was measured by the release of the 32P-labeled single-stranded (ss) oligonucleotide from the double-stranded (ds) DNA substrate shown in the inset and described in the text. The gel and graph show the amount of 37-mer oligonucleotide released from the DNA substrate in reaction mixtures containing increasing concentrations of gp4Δprimase (filled circles on the graph) or WT gp4 (open circles). D, strand displacement activity of gp4Δprimase was measured in a minicircle assay. A minicircle DNA (inset) containing a replication fork was prepared as described previously and used as a primer/template (28). The reactions contained the minicircle DNA, the four dNTPs with [α-32P]dGTP, gp5/ trx, and gp4Δprimase or WT gp4. In the control reaction gp4 was omitted. Incorporation of [α-32P]dGTP into the minicircle DNA is shown as the histogram.

**DNA Unwinding Activity and Strand Displacement Synthesis with gp5/trx**—Once loaded onto ssDNA, gp4 translocates in a 5’ to 3’ direction and unwinds dsDNA using energy obtained from dTTP hydrolysis. The dTTP hydrolysis activity of gp4Δprimase and WT gp4 are the same (27). In this section we compare the unwinding activity of both proteins.

The DNA substrate for the unwinding assay was prepared by annealing a 32P-labeled 37-mer oligonucleotide with a circular M13 DNA such that a 22-nt double-helical fragment is formed, as well as a 5’ tail of 15 nucleotides (Fig. 5C, inset). Reaction mixtures containing the DNA substrate described above, dTTP, and different concentrations of gp4Δprimase or WT gp4 were incubated for 10 min and then stopped and analyzed on the gel (Fig. 5C, gel). Intensities of ssDNA and dsDNA bands were measured in a PhosphorImager. At WT gp4 of 0.24 μM, 50% of the 37-mer is displaced from the DNA substrate. In contrast, we estimate that 1 μM gp4Δprimase is required for unwinding of the identical amount of DNA (Fig. 5C, graph). gp4Δprimase is five times less active than WT gp4 in unwinding DNA.

The low binding affinity of gp4Δprimase to ssDNA and the resultant low unwinding activity account for the lack of detectable leading strand DNA synthesis measured in the single-molecule assay (Fig. 3). In the single-molecule assay, we can simultaneously monitor only several individual replicons. Consequently, the protein and DNA concentrations used are relatively low, making it difficult to investigate the activity of proteins with low binding affinity to DNA. Therefore, we have examined the ability of gp4Δprimase to mediate leading strand synthesis using a DNA minicircle assay (18). The minicircle, depicted in the inset of Fig. 5D, consists of a 70-bp circular dsDNA with a replication fork and a 5’ tail onto which gp4 can assemble. The incorporation of [32P]dGMP allows for the measurement of leading strand synthesis. Using this assay, we do detect DNA synthesis by the gp4Δprimase-gp5/trx complex (15% of that measured for WT gp4).

**Small Angle X-ray Scattering Structure of the Leading Strand Complex**—We have used SAXS to analyze a solution structure of the complex between gp4Δprimase, gp5/trx, and a primer/template. The SPR experiments presented in Fig. 2 show that stable complexes of the two proteins form in the presence of a primer/template, ddATP and the next incoming nucleotide (Fig. 2) (8). Therefore, samples for SAXS were prepared in an analogous manner.

gp5/trx bound to a primer/template in the presence of ddATP and ddGTP was mixed with gp4Δprimase previously incubated with 15-mer single-stranded oligonucleotide in buffer containing β,γ-methylene dTTP. The 15-mer was included to ensure a uniform oligomerization pattern of gp4Δprimase (Fig. 5B). The SAXS spectrum acquired for the complex described above and the corresponding Guinier plot are presented in Fig. 6 (A and B, respectively). Linearity of the...
very small angle part of the spectrum \((q^2 \geq 0.00064 \text{ Å}^{-2})\) indicates no aggregation. An upward deviation from linearity in the wider angle portion of the spectrum \((>0.004 \text{ Å}^{-2})\) corresponds to more pronounced aberrations from internal symmetry. The calculated hydrated radius of gyration \(\langle R_g \rangle\) for the leading strand complex is 60.5 Å (supplemental Table S3). We also carried out control SAXS measurements of samples containing gp5/trx-primer/template or gp4Δprimase-ssDNA. The calculated \(R_g\) values are 34 and 43 Å, respectively. The distance distribution function \(p(r)\) measured for the leading strand complex is presented in Fig. 6A. To obtain a reliable result of the maximum diameter \(D_{max}\), we used a search approach. In this method, \(p(r)\) was calculated from SAXS data when \(D_{max}\) was set as a variable in the range of 100–300 Å with increments of 10 Å. Evaluation parameters were analyzed, and the values of \(D_{max}\) that showed the best overall behavior and GNOM statistics were chosen for further analysis (supplemental Fig. S2). The \(p(r)\) of the leading strand complex shows a \(D_{max}\) of 180 Å. The bell-like shape of the \(p(r)\) indicates a compact sphere-like structure. The \(D_{max}\) values for the samples containing gp4Δ primase-ssDNA and gp5/trx-primer/template are 126 and 105 Å, respectively (supplemental Table S3). We also used SAXS to investigate the interactions between gp5/trx bound to a primer/template with WT gp4, gp4ΔZBD, or gp4ΔC. Attempts to obtain structures for the above complexes were unsuccessful because of aggregation of the proteins at high protein concentration.

We have used the software GASBOR to reconstruct \textit{ab initio} 10 structural models of the leading strand complex (24). The final averaged model, the first low resolution structure of the complex between gp5/trx, and DNA helicase, is shown in Fig. 6D. The crystal structures of the hexameric DNA helicase (33) and gp5/trx bound to a primer/template (6) were docked into the SAXS envelope using the MultiFit method in the program Chimera. The model of the 17-amino acid C-terminal tail of gp4 that did not diffract in crystal was built in PyMol and is displayed as a yellow surface. Residues from the basic loading patch are colored in white. \(E\), choreography of a replication fork modeled onto the leading strand complex. The residues from the subunit interface \(\beta\)-hairpin with Phe-523 are shown as the orange patch at the front surface of DNA helicase (see “Discussion”).

**DISCUSSION**

During leading strand synthesis, gp5/trx and gp4 make numerous interactions. gp5/trx joins the replisome by binding to the C-terminal acidic tail of gp4 via its basic loading patch (Fig. 7A). Alternatively, gp5/trx first binds the C-terminal tail through a basic exchange patch (Fig. 7B), but it must still switch its binding to the loading patch to initiate DNA synthesis (11). Elimination of the basic charges in the exchange patch does not affect strand displacement synthesis (9). Once leading strand synthesis is initiated, a highly stable complex of gp5/trx and DNA helicase allows for binding of the C-terminal tail of the helicase (yellow) to a basic patch on the front of the polymerase (white).
DNA Polymerase-DNA Helicase Interaction

FIGURE 7. The specific interactions of gp5/trx and gp4 coordinate each step in DNA replication. A, the gp4 assembles as a hexamer on the lagging strand DNA. gp5/trx is recruited to the replication fork by an initial interaction with the acidic C-terminal tail of gp4 through its loading patch containing basic residues. B, gp5/trx can also initially bind to the C-terminal tail of gp4 via its exchange patch located within the thioredoxin-binding domain (TBD) and containing two basic loops. C, the interaction of the loading patch of gp5 with the C-terminal tail of helicase facilitates the loading of gp5/trx to the primer/template, and the interaction is maintained during leading strand synthesis. gp5/trx and gp4 also interact through the helicase domain of gp4 and a part of the surface of the polymerase domain of gp5/trx. D, the C-terminal tails of the gp4 hexamer not only bind any dissociating gp5/trx, but they also accommodate additional gp5/trx through interactions with the exchange patch of gp5. Any of these gp5/trx can be reloaded onto the primer/template without involvement of the loading patch.

approximately every 5 kb. If gp5/trx does dissociate into solution, it can still be replaced by a gp5/trx already bound by its exchange patch to a C-terminal tail of one of the subunits of the hexameric gp4 (Fig. 7D). Thus the interaction of the C-terminal tail with the exchange patch of gp5 increases the processivity to >17 kb (9).

Lacking to date has been an identification of the stable complex formed between gp5/trx and gp4 during leading strand synthesis. This complex that mediates leading strand synthesis is extremely stable with a half-life of more than 10 min (8). The binding between gp4 and ssDNA is stable, with a dissociation rate of 0.002 s⁻¹ (35). However, the complex of gp5/trx, in the absence of gp4, readily dissociates from a primer/template with a dissociation constant of 0.2 s⁻¹ (36). Consequently, there is an interaction of gp5/trx with gp4 that accounts for the association observed during leading strand synthesis.

In the current study we show that the stable complex is mediated through the interaction of the helicase to the palm domain of gp5 and an electrostatic binding of the C-terminal tail of the helicase to the loading patch of gp5. The ZBD and RPD are not directly involved in binding as indicated by nearly identical Rmax values measured using SPR for WT gp4, gp4ΔZBD, and gp4Δprimase, as well as the lack of binding measured with gp4Δhelicase. The primase domain of gp4 is involved in formation of the leading strand complex by stabilizing the helicase. gp4Δprimase associates with gp5/trx-primer/template in a similar way as WT gp4; however, its dissociation from the complex is faster. Removal of the ZBD from gp4 has a minimal influence on leading strand synthesis as measured in the single-molecule assay. Removal of both the ZBD and the RPD results in a significant reduction of leading strand synthesis indicative of the major role of the RPD in stabilization of the helicase. The sedimentation velocity measurements support the above conclusions and show that removal of the primase domain affects the dynamic equilibrium between various oligomeric forms of the helicase. A similar stabilization effect of the primase on the helicase can be observed in the crystal structure of B. stearothermophilus primase/helicase complex (25). In the structure, the three helicase binding domains of the primase stabilize a conformation of the N-terminal domain of the helicase.

Additionally, the primase is important for gp4 binding to ssDNA and for dsDNA unwinding. gp4Δprimase binds the ssDNA ~3-fold less tightly and displays five times less unwinding activity than WT gp4. The reduction in DNA binding and unwinding activities is due to the removal of the RPD from gp4. We showed previously that gp4ΔZBD retains full helicase activity and displays ssDNA binding comparable with the WT protein (16). It was proposed earlier that the primase facilitates loading of gp4 onto ssDNA (32). The five-step model involves the fast initial binding of the primase to the primase recognition sequence in DNA followed by a rapid conformational change in gp4 that triggers ring opening, movement of the DNA into the central channel of the helicase ring, and ring closure. The current study is consistent with this model.

We propose that an electrostatic interaction of the C-terminal tail of the helicase to the loading patch located on the front of the polymerase is maintained during leading strand synthesis. Removal of the C-terminal tail of gp4 decreases the binding of gp4 to the leading strand complex. In addition, elimination of the interaction described above abolishes initiation of strand displacement synthesis and reduces the rate of ongoing synthesis by 50% (11). We estimate that the interaction between the C-terminal tail of the helicase and a basic patch on gp5 accounts for 30% of the binding between gp4 and the leading strand com-
plex as evidenced by SPR measurements with gp4ΔC. The remaining 70% of binding is due to the interaction of the helicase and the palm domain of gp5. We measured using SPR that gp4ΔC-primase binds to the leading strand complex at 30% of the level observed with WT gp4. Therefore, we estimate that the presence of the primase stabilizes the helicase, contributing 40% of the binding between gp4 and gp5/trx during leading strand synthesis.

We present the first low-resolution structure of the complex between DNA polymerase and DNA helicase solved using SAXS. In the SAXS model, the protein-protein interface includes the gp5 surface located at the palm domain in the close proximity to helices: R, Q, and G1 and the cleft between the two neighboring gp4 subunits of the hexameric ring (Fig. 6E). The relative orientation of gp5/trx and the DNA helicase in the complex allows for binding of the C-terminal tail of the helicase to a basic patch on the front of the polymerase. In the SAXS model a primer/template bound to gp5/trx (displayed in black in Fig. 6E) is positioned such that the extension of the 5'-end of the ssDNA template would position it in the close proximity to the subunit interface β-hairpin that contains residue Phe-523 (orange patch at the front surface of DNA helicase). We demonstrated earlier that the subunit interface β-hairpin, specifically Phe-523, interacts directly with the extruded DNA leading strand during the process of DNA unwinding (17). The lagging strand passes through the central channel in the hexameric ring of DNA helicase, making interactions with the residues in the central β-hairpins located at the inner surface of the channel. At the replication fork, a 5’ to 3’ forward motion of gp4 relative to the lagging strand to which it is bound unwinds the duplex DNA that it encounters. In the model, the extruded DNA strand has the correct polarity and position to bind at the DNA-binding crevice of gp5 such that the 3'-terminus of the leading strand primer is situated in the active site. This configuration of the replication fork is stabilized by the two interactions between gp5/trx and DNA helicase described in the present study.

gp4 has been crystallized in three different oligomeric forms. The structure of gp4ΔZBD revealed a heptameric arrangement of the ring subunits (29). In contrast, gp4Δprimase was crystallized as a hexamer (33). Deletion of an additional 31 amino acids from the N terminus of the gp4Δprimase results in the formation of a helical filament with six monomers per helical turn (37). Here, we have identified yet another oligomeric form of gp4. The EM analysis of WT gp4 and gp4Δprimase revealed the presence of octameric rings in the samples. Similar structural diversity has been observed with proteins involved in DNA recombination. In bacterial homologous recombination, RecA protein is a key enzyme that mediates a homology search and exchange between two DNA molecules. RecA protein exists in two different oligomeric forms. In the crystal E. coli RecA forms helical filaments (38), but the EM studies of RecA under similar conditions revealed the presence of hexameric rings (39). The archaeal homolog of RecA, Sulfolobus solfataricus RadA protein, can form octameric rings or helical filaments (40). The Pyrococcus furiosus RadA protein (PRad51) was crystallized as a heptamer (41), and EM studies revealed hexameric filaments (42). The human Dmc1 protein is a RecA homolog that catalyzes homologous pairing during meiosis. Structural analysis of Dms1 revealed that this protein forms both heptameric and octameric rings (43, 44), as well as helical filaments with a different number of monomers per helical turn depending on the DNA template to which it is bound (45). What is the functional relevance of the octameric gp4? Is it enzymatically active, or perhaps various oligomerization patterns are simply a natural consequence of flexible subunit interfaces? Structural similarities between gp4 and the recombination proteins bring an intriguing possibility of the involvement of gp4 in DNA recombination and repair.

In conclusion, we have identified the interaction between the DNA polymerase and DNA helicase that is essential for the high processivity of leading strand synthesis, and described it in the context of other specific interactions that govern the interplay between these two enzymes. We hope that understanding of the replication process in bacteriophage T7 will facilitate studies in more complex systems.

Acknowledgment—We thank Steven Moskowitz for assistance in preparation of figures.

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Arkadiusz W. Kulczyk, Barak Akabayov, Seung-Joo Lee, Mihnea Bostina, Steven A. Berkowitz and Charles C. Richardson

*J. Biol. Chem.* 2012, 287:39050-39060.
doi: 10.1074/jbc.M112.410647 originally published online September 12, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.410647

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