DNA polymerases responsible for copying genomic DNA require high processivity to incorporate thousands of nucleotides without dissociating from the DNA (1–3). In most cases, a DNA polymerase achieves high processivity by utilizing accessory proteins that act as a sliding clamp that encircles the DNA to tether the polymerase to a primed DNA template (4–6). For example, the DNA polymerase of bacteriophage T7 adopts the host protein E. coli thioredoxin (trx) as a processivity factor. After infecting its host, E. coli, bacteriophage T7 induces the synthesis of a replicative DNA polymerase, the product of gene 5 (7). Gene 5 protein alone is a distributive enzyme, dissociating from a primed DNA template after incorporation of only a few nucleotides (8). The gene 5 protein achieves high processivity by forming a 1:1 complex (Kd ~5 nM) with E. coli thioredoxin (8–11). The complex of T7 gene 5 DNA polymerase and thioredoxin is designated as gp5/trx, also known as T7 DNA polymerase. Thioredoxin allows the gene 5 protein to incorporate thousands of nucleotides per polymerization cycle, a result of an 80-fold increase in the affinity of gp5/trx for the 3' terminus of the primer-template (8, 9). Thioredoxin also markedly increases the 3'-5' double-stranded DNA exonuclease activity of the polymerase, but does not affect the single-stranded DNA exonuclease activity (8, 12, 13).

The crystal structure of gp5/trx has been determined at 2.2 Å resolution with the polymerase captured in a polymerization mode (Ref. 17 and Fig. 1). T7 gene 5 protein is a member of the pol I family of DNA polymerases with three subdomains: palm, fingers, and thumb. The three subdomains together form a DNA binding groove with the palm forming the base of a cleft, and the fingers and thumb creating a wall on each side. In this structure thioredoxin is bound to the polymerase at a flexible loop extending from the thumb and is rotated slightly up and away from the cleft in which the primer-template lies. Previous biochemical studies have characterized the domain in gene 5 protein that is responsible for binding thioredoxin. An amino acid alignment of gene 5 protein with homologous regions of the Klenow fragment of E. coli DNA polymerase I revealed a 71 amino acid extension between α-helices H and H1 of thethumb that is absent from the Klenow fragment (18). Mutations within this domain affect the ability of the polymerase to bind thioredoxin (19). Furthermore, insertion of this domain into the corresponding region of the thumb in the Klenow fragment results in a chimeric DNA polymerase that can bind thioredoxin and achieve higher processivity (20).

The precise molecular mechanism by which thioredoxin increases the processivity of gp5 is not known. However, unlike the processivity factor of E. coli DNA polymerase III, thioredoxin does not appear to encircle the DNA as a clamp. It is likely that in the crystal structure the polymerase-DNA complex has been captured in a non-processive mode. In a proces-
Polymerase-Thioredoxin Covalent Complex

Bacterial Strains and DNA—Bacterial strain BL21(DE3), used to express wild-type thioredoxin, was purchased from Invitrogen. E. coli A307 (HrfC, trxA307) was a gift from Stan Tabor (Harvard Medical School). E. coli trxA307 (DE3) was constructed from E. coli A307 using a DE3 lysogenization kit from Novagen. Using this kit, E. coli A307(DE3) was infected with a ΔDE3 prophage carrying the gene for T7 RNA polymerase under lacUV5 control so that expression of cloned genes having a T7 promoter could be induced in the presence of IPTG. T7Δ5 phage, lacking gene 5, were a gift from Stan Tabor. M13 mp18 bacteriophage were a gift from Kajal Chowdhury (Harvard Medical School). M13 phage were grown and purified as described (33). Single-stranded M13 DNA were obtained from Midland Certified Reagent Co. Plasmids pOP5-3, pTrx-3, and pGPI-2, vectors having wild-type T7 gene 5, E. coli trxA, and T7 gene 1, respectively, were gifts from Stan Tabor. Plasmid pET7-7, the parent vector of pGPl-3 and pTrx(C55S)-1, was a gift from Stan Tabor. Plasmid pET-24a, the parent vector of pTrxA and pTrxA(c55s)-2, was purchased from Novagen.

Mutagenesis of T7 Gene 5—Plasmid pGPl5(T327C) was constructed by mutagenesis of T7 gene 5 within pGPl-5 using an "overlap extension" method (34). The mutagenesis required three separate PCR reactions using PfuTurbo DNA polymerase (Stratagene). The first PCR reaction used the primers DJ4 (5'-GAAGGTTTATCAAGCATTACGTTCAACCTGGGCAAGGACGACTGGA-3') and BCMP97 (5'-GCATTGACCAAACCTGGCAAAAG-3') to generate a 5' fragment of T7 gene 5 that contains a codon that corresponds to a Thr-327 deletion. The altered codon of primer DJ4 is underlined. The second PCR fragment containing the same codon was generated in a second PCR reaction using primers DJ3 (5'-AGTACGTGGTCTGGTCTCTTACTGCCCAGTGTGATGGTTGTATTACCC-3') and JH8 (5'-GGCGAAGCCTAGAAGTG-3'). The 5' and 3' fragments were purified by agarose gel electrophoresis and then used in a final PCR reaction. These fragments overlap and generate a longer fragment when amplified with primers BCMP97 and JH8. The final PCR product was sequenced on an agarose gel, digested with StyI and MfeI, and ligated into corresponding sites on plasmid pGPl-3 to create pGPl5(T327C). The desired clone was confirmed by DNA sequencing.

Mutagenesis of E. coli trxA—Plasmid pTrxA(C55S) was constructed by mutagenesis of E. coli trxA of plasmid pTrx-3 using a "Megaprimmer" method (35). Plasmid pTrx-3 contains a copy of wild-type E. coli trxA. The mutagenesis required two separate PCR reactions using PfuTurbo DNA polymerase (Stratagene). The first PCR reaction used primer A (5'-GAGTGGTGGTGCCTGGTCTCATCAGGCTTTTGGCCTA-3') and primer B (5'-GCTTCTAACGATCCGCAGGTGCTTT-3') to generate a 5' fragment of the trxA gene having a codon that corresponds to a Cys-35 to Ser alteration. The altered codon of primer A is underlined. This 5' fragment was purified by agarose gel electrophoresis and then used in a final PCR reaction with the primer C (5'-GTTGGTGGCGCCCTATGAGCCATAAATTTACAC-3') to generate a full-length copy of the trxA gene with the desired mutation. The final PCR product


The structure of reduced thioredoxin in gp5/trx is very similar to that of oxidized thioredoxin (17), yet only reduced thioredoxin binds to gene 5 protein (12). In the crystal structure of gp5/trx the thioredoxin binding loop of gp5 wraps around the base of thioredoxin, burying the active site cysteines (17). Thus, it is not surprising that the active site cysteines are not required for their reducing power when thioredoxin binds gp5 (29). Both Cys-32 and Cys-35 can be replaced with residues that abolish the ability of thioredoxin to undergo oxidation-reduction reactions, but these altered forms of thioredoxin can form functional polymerase-thioredoxin complexes in vitro, albeit with a reduced binding affinity. These results show that the active site residues of thioredoxin only function in binding thioredoxin to the polymerase. The three-dimensional structure of gp5/trx supports these findings, revealing that thioredoxin Cys-32 is exposed to the protein-protein interface and hydrogen bonds with Thr-327 of the polymerase thumb (17). Thr-327 of the polymerase selects for reduced thioredoxin through its hydrogen bond with the sulfhydryl group of Cys-32. This interaction effectively decreases the polarity of Thr-327 within the hydrophilic subunit interface and thus explains the requirement for reduced thioredoxin for binding. Cys-32 of oxidized thioredoxin cannot participate in a hydrogen bond with Thr-327 of gp5 because it forms a disulfide linkage with Cys-35. In the present study we have substituted Thr-327 of the polymerase thumb with cysteine (gp5(T327C)) so that it can react with Cys-32 of thioredoxin to facilitate a mixed disulfide between the two proteins. We have used the covalently linked complex (gp5-S-S-trx) to examine processivity and to determine if there is a requirement for thioredoxin to dissociate when the polymerase recycles from one template to another.

EXPERIMENTAL PROCEDURES

Materials

At a replication fork, gp5/trx interacts with the T7 gene 4 helicase-primease (14) and the T7 gene 2.5 single-stranded DNA-binding protein (40) to mediate coordinated leading and lagging strand DNA synthesis (15, 16). Like E. coli DNA polymerase III, gp5/trx synthesizes both strands processively (15, 16). The leading strand polymerase synthesizes DNA at the replication fork in a continuous manner, while the lagging strand polymerase replicates Okazaki fragments in a discontinuous manner. It is postulated that DNA polymerase III must rapidly recycle from the DNA and β-clamp upon completion of an Okazaki fragment to associate with another pre-assembled β-clamp for processive synthesis of the next Okazaki fragment (3). During coordinated DNA synthesis by the T7 replisome, the lagging strand gp5/trx also recycles from a completed Okazaki fragment to a new primer (15, 16). However, it is unclear whether gp5 dissociates from thioredoxin as the polymerase recycles. In this study, we have examined the fate of thioredoxin during recycling by forming a covalent linkage between the polymerase and thioredoxin.

Thioredoxin is a versatile protein found in all species, serving as a cofactor to reduce disulfide bonds in many proteins (22, 23). Among its many functions it acts as a hydrogen donor for the enzyme ribonucleotide reductase. The activities of thioredoxin have been attributed to two active site cysteines that can form a disulfide linkage between their sulfhydryl groups or can participate in reversible oxidation-reductions with other proteins. The thioredoxin active site cysteines are part of a conserved sequence, Cys-Gly-Pro-Cys (residues 32–35) located in a loop that is partially exposed to the surface of the protein (24, 25). This loop participates in a hydrophobic surface that is responsible for binding to protein substrate (26). Once bound to a protein having a disulfide bond, residues Cys-32 and Cys-35 of thioredoxin act together to reduce their target substrate. Cys-32 acts as a nucleophile to form a covalently mixed disulfide with the target protein in the transition state (22, 23, 27). Cys-35 then resolves this intermediate mixed disulfide to yield the reduced target protein (27).

The structure of reduced thioredoxin in gp5/trx is very similar to that of oxidized thioredoxin (17), yet only reduced thioredoxin binds to gene 5 protein (12). In the crystal structure of gp5/trx the thioredoxin binding loop of gp5 wraps around the base of thioredoxin, burying the active site cysteines (17). Thus, it is not surprising that the active site cysteines are not required for their reducing power when thioredoxin binds gp5 (29). Both Cys-32 and Cys-35 can be replaced with residues that abolish the ability of thioredoxin to undergo oxidation-reduction reactions, but these altered forms of thioredoxin can form functional polymerase-thioredoxin complexes in vitro, albeit with a reduced binding affinity. These results show that the active site residues of thioredoxin only function in binding thioredoxin to the polymerase.
Polymerase-Thioredoxin Covalent Complex

was purified on an agarose gel, digested with NdeI and HindIII, and ligated into corresponding sites on plasmid pET-7 to create pTrx (C35S)-1, the plasmid used to express trx(C35S) in the presence of ampicillin for purification. The restricted fragment containing the trxA gene was also ligated into corresponding sites on plasmid pET-24a to create pTrx(C35S)-2, the plasmid used for determining plating efficiencies in the presence of kanamycin. Plasmid pTrxA was used to express wild-type thioredoxin in the presence of kanamycin. It was created by PCR of trxA of plasmid pTrx-3 using primer A and primer C, followed by digestion with NdeI and HindIII, and then ligation into corresponding sites on plasmid pET-24a. The desired clones were confirmed by DNA sequencing.

Methods

Plating Efficiencies—Plating efficiencies of T7/5 phage were measured on E. coli A307 (Δtrax) harboring either plasmid pTrx-C, gp5P(T327C), pGP5(T327C), pET-24a, pTrxA, pTrx(C35S)-2, or a combination of two of these plasmids (Table II). Cells having plasmids pTrx-C, gp5P(C), and pGP5(T327C) were selected for ampicillin resistance. Cells harboring plasmids pET-24a, pTrxA, and pTrx(C35S)-2 were selected for kanamycin resistance. 10-fold serial dilutions of T7/5 phage (100 μl) were mixed with a 100-μl plating culture and 3 ml of top agar (1% tryptone, 0.5% yeast extract, 5% NaCl, 1.5% agar, pH 7.0) containing 200 μl of reaction mixtures. The 3H-labeled plasmid was separated from free gp5(T327C) using a 200 μl aliquot from each reaction mixture. Reactions involving poly(dA)350-oligo(dT)25 were incubated at 37°C for 5 min and stopped by heating at 75°C for 5 min, followed by re-annealing at room temperature for 30 min. The labeled DNA was extracted with phenol-chloroform and then purified by passing through BioSpin 6 columns (Bio-Rad) to remove free nucleotides. Radioactivity in single- and double-stranded DNA was determined using a Packard liquid scintillation counter.

The double-stranded DNA exonuclease activity of gp5/S-trx and gp5p-S-trx were assayed using procedures modified from those previously described (37, 39). Uniformly 3H-labeled double-stranded M13 DNA was prepared by annealing a 17-nt primer (–40 primer) to 50 ml Tris-CI (pH 7.5), 50 μM NaCl. Annealing reactions were incubated at 75°C for 5 min followed by 30 min at room temperature. For polymerase assays using linear DNA templates, poly(dA)350 (2 μM) was annealed to an oligo(dT)25 primer in a 1:1 molar ratio at 80°C for 5 min. Mesosporobacter [poly(dT)25] and [3H]DNA hydrolyzed.

The double-stranded DNA exonuclease assays (200 μl) contained 50 mM Tris-CI (pH 7.5), 10 mM MgCl2, 0.1 mM DTT, 50 μM NaCl, 16 mM gp5/S-trx or gp5p-S-S-trx, and 8 μM 3H-labeled, double-stranded M13 mp8 DNA. Reaction mixtures were incubated at 37°C, and 20 μl aliquots were removed at 1-min intervals from 1 to 10 min and stopped by addition of EDTA to a final concentration of 25 mM. Aliquots were spotted on DE81 ion exchange filters, and unincorporated radiolabeled nucleotides were washed away with three successive 10-min washes in 300 mM ammonium formate (pH 8.0). Filters were dried under a heat lamp, and the amount of [3H]DNA incorporated was measured by liquid scintillation counting to calculate the amount of DNA hydrolyzed.

The double-stranded DNA exonuclease activity contained 40 μM of SSB protein per 200-μl reaction mixture. Stationary-phase cultures of M. thermoautotrophicus (ATCC 7953) were grown in Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, and 10% glycerol. After loading the diluted mixture on the HiTrap heparin column using a BioCad Sprint System (Perseptive Biosystems Inc.), the column was washed with 200 mM NaCl in Buffer H to remove free gp5(S-S-trx). gp5p-S-S-trx was separated from free gp5(T327C) using a 200-800 mM NaCl continuous gradient in Buffer H over 60 min. Under these conditions, gp5p-S-S-trx eluted at 610 mM NaCl and was determined to be greater than 95% pure by analysis on a 4%–20% SDS-PAGE gel. Purified gp5p-S-S-trx was stored at –12°C in a buffer containing 40 mM potassium phosphate (pH 7.4), 0.1 mM DTT, 1 mM EDTA, and 50% glycerol. Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay kit and bovine serum albumin as a standard.

DNA Polymerase Assays—DNA polymerase activity was measured by procedures modified from those previously described (3, 37, 38). The DNA polymerase assay (300 μl) for M13 DNA contained 50 mM Tris-CI (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 0.1 mM DTT, 50 mM kanamycin, and 100 μM of each dNTP. The DNA synthesis reactions contained 64 nM gp5p/trx and 4 nM of either gp5p/trx or gp5p-S-S-trx. The polymerase reactions were carried out at 37°C. Aliquots (20 μl) were removed at the times indicated and stopped by addition of EDTA to a final concentration of 25 mM. Aliquots were spotted on DE81 ion exchange filters, and unincorporated radiolabeled nucleotides were washed away with three successive 10-min washes in 300 mM ammonium formate (pH 8.0). Filters were dried under a heat lamp, and the amount of [3H]TMP incorporated was measured by liquid scintillation counting. Assays to study the effect of E. coli SSB protein on polymerase activity contained 64 nM gp5p/trx and 40 μM SSB protein per 300 μl of reaction mixture. Assays to monitor polymerase activity on poly(dA)350-oligo(dT)25 were similar for those with single-stranded M13 DNA except poly(dA)350-oligo(dT)20 was added at a concentration of 200 nM, resulting in a 50-fold molar excess of DNA over polymerase. Reactions involving poly(dA)350-oligo(dT)20 were incubated at 25°C.

M13 DNA (100 nm) was used for polymerase assays by annealing with a 17-nt primer (–40 primer) to 50 mM Tris-CI (pH 7.5), 50 mM NaCl. Annealing reactions were incubated at 75°C for 5 min followed by 30 min at room temperature. For polymerase assays using linear DNA templates, poly(dA)350 (2 μM) was annealed to an oligo(dT)25 primer in a 1:1 molar ratio at 80°C for 5 min. Mesosporobacter [poly(dT)25] and [3H]DNA hydrolyzed.

The double-stranded DNA exonuclease assays (200 μl) contained 50 mM Tris-CI (pH 7.5), 10 mM MgCl2, 0.1 mM DTT, 50 mM NaCl, 16 mM gp5p/trx or gp5p-S-S-trx, and 8 μM 3H-labeled, double-stranded M13 mp8 DNA. Reaction mixtures were incubated at 37°C, and 20 μl aliquots were removed at 1-min intervals from 1 to 10 min and stopped by addition of EDTA to a final concentration of 25 mM. Aliquots were spotted on DE81 ion exchange filters, and washed with three successive 10-min washes of 300 mM ammonium formate (pH 8.0). Filters were dried under a heat lamp, and the amount of [3H]DNA remaining was determined using a Packard liquid scintillation counter to calculate the amount of DNA hydrolyzed.

Reaction mixtures for single-stranded DNA exonuclease assays were similar as for the reactions for double-stranded M13 DNA except reactions contained 5 μM gp5p/trx or gp5p-S-S-trx and –8 μM 3H-labeled, single-stranded M13 mp8 DNA. Assays to study the effect of SSB protein on polymerase activity contained 80 nM gp5p/trx and 80 μM SSB protein per 200 μl of reaction mixture.

Processivity Assays—Processivity assays were carried out on a single-stranded M13 DNA primed with a 5’-32P-labeled 17-nt primer (–40 primer) in the absence or presence of SSB protein by procedures modified from those previously described (3, 37). DNA synthesis reactions contained 80 nM gp5p/trx or gp5p-S-S-trx and 80 μM 3H-labeled, single-stranded M13 mp8 DNA. Assays to study the effect of SSB protein on polymerase activity contained 80 nM gp5p/trx and 80 μM SSB protein per 200 μl of reaction mixture.
to introduce a disulfide bond between the two proteins. Our interest was to determine the effect a covalent linkage between the two subunits has on gp5/trx activity. Therefore, we replaced Thr-327 of gene 5 protein with a cysteine residue that should be in a position to form a disulfide bond with thioredoxin Cys-32. Cys-35 of thioredoxin was also replaced with serine so that it would not resolve the mixed disulfide.

Although the active site cysteines of thioredoxin are not required for their redox potential when bound to gene 5 protein, thioredoxin must be in a reduced form (28, 29). Oxidized thioredoxin does not bind to the polymerase. Therefore, a reducing agent such as DTT is also required in reactions containing gp5/trx (28). In addition T7 gene 5 protein has a number of cysteines on its surface (17), and the requirement for a reducing environment most likely derives from their presence. In the absence of reducing agent, these residues form intermolecular disulfide bonds leading to protein aggregation and loss of enzyme activity.\(^2\) Therefore, in the present study we routinely included DTT in all reactions containing gp5/trx. Its presence does not interfere with the formation of the disulfide, gp5-S-S-trx, as shown below.

To prepare a linked complex of gene 5 protein and thioredoxin, we initially purified gp5(T327C) and trx(C35S) to apparent homogeneity from E. coli cells overexpressing the mutant genes as described under “Experimental Procedures.”\(^3\) E. coli strains used to overexpress gp5(T327C) and trx(C35S) lacked trxA so that wild-type thioredoxin would not contaminate the protein preparations. gp5(T327C) (20 \(\mu\)M) was mixed with an 8-fold molar excess of trx(C35S) in 40 mM potassium phosphate (pH 7.4), 25 mM NaCl, 1 mM DTT, 1 mM EDTA, 50% glycerol, and incubated at 0 \(^\circ\)C overnight to facilitate intermolecular disulfide formation. The resulting covalently linked complex was purified to apparent homogeneity using heparin chromatography (see “Experimental Procedures”).

Interestingly, gp5-S-S-trx has maintained a stable disulfide even though DTT was present during purification. Apparently, the tight association of the mutant subunits excludes the reducing agent from their binding surface. To demonstrate the
presence of a covalent linkage in gp5-S-S-trx, the complex was analyzed and compared with wild-type gp5/trx on a denaturing polyacrylamide gel in the presence of SDS (Fig. 2). The complex of wild-type gp5 and trx is resolved into its two components either in the presence (lane 5) or absence (lane 7) of DTT. However, gp5(T327C)/trx(C35S) migrates as a complex even in the presence of SDS provided no reducing agent is present (lane 8), indicating the presence of a disulfide linkage. Only if DTT is present during denaturation does the complex resolve into gp5(T327C) and trx(C35S) (lane 6). To ensure that any disulfide bonds already formed and buried at the protein-protein interface were not reduced by any residual DTT during the SDS-promoted denaturation of the complex, gp5-S-S-trx was treated with NEM (lane 8). NEM alkylates free sulfhydryl groups on both DTT and proteins. NEM treatment also prevents unwanted disulfide bonds between denatured proteins. Efforts to make a covalent linkage between gp5(T327C) and wild-type thioredoxin were not pursued since the presence of a disulfide bond between the two proteins could not be detected (data not shown). Apparently, Cys-35 of thioredoxin disrupts the disulfide bond prior to or when the two subunits are denatured during gel analysis. Therefore, we have used trx(C35S) as the processivity factor to form a covalent complex with gp5(T327C) and characterized its activity below.

DNA Polymerase Activity of gp5-S-S-trx—We first compared the polymerase activity of gp5-S-S-trx to that of gp5/trx on primed, single-stranded M13 DNA. In the experiment shown in Fig. 3A, the M13 DNA template was present in a 4-fold molar excess over the gp5-S-S-trx complex. The specific activities from the linear range of the data are presented in Table I. The initial rate of synthesis catalyzed by gp5-S-S-trx is ~5-fofold lower than that observed with gp5/trx. Additionally, the extent of synthesis over the 30-min incubation period is considerably lower with the covalent complex, gp5/trx is able to replicate a majority of the M13 molecules available in the reaction mixture, whereas gp5-S-S-trx replicates a much smaller fraction of the DNA template.

M13 DNA has many sites of secondary structure along its length that cause gp5/trx to pause during DNA replication (30).
DNA polymerase activities were determined as described under “Experimental Procedures.” Reaction mixtures were incubated with either primed-M13 single-stranded DNA or poly(dA)350-oligo(dT)25. DNA polymerase activities were measured by the incorporation of [3H]TMP over time in the presence or absence of E. coli single-stranded DNA binding protein.

<table>
<thead>
<tr>
<th>Gene 5 protein/thioredoxin</th>
<th>M13 ssDNA</th>
<th>poly(dA)350-oligo(dT)25</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp5/trx</td>
<td>22,000</td>
<td>62,000</td>
</tr>
<tr>
<td>gp5-S-S-trx</td>
<td>4000</td>
<td>61,000</td>
</tr>
<tr>
<td>gp5/trx + SSB protein</td>
<td>78,000</td>
<td>62,000</td>
</tr>
<tr>
<td>gp5-S-S-trx + SSB protein</td>
<td>81,000</td>
<td>62,000</td>
</tr>
</tbody>
</table>

* Specific activities were determined as nmol of TMP incorporated per min per mg of protein.

Since *E. coli* SSB protein can eliminate regions of secondary structure (31) we also examined DNA synthesis catalyzed by the two complexes in the presence of this protein (Fig. 3B). SSB protein is known to stimulate the elongation rate of DNA synthesis by gp5/trx on primed single-stranded M13 DNA (9, 30). In the presence of SSB protein both complexes are able to replicate nearly all the available M13 molecules. Additionally, both the rate and extent of DNA synthesis are identical for both gp5/trx and gp5-S-S-trx (Fig. 3B and Table I). These results suggest that the presence of the covalent linkage between polymerase and its processivity factor prevents the polymerase from progressing through sites of secondary structure. It seems unlikely that the stimulating effect of SSB protein is due to an increased binding affinity of the covalent complex with the primer-template since it has been shown previously that SSB protein does not stimulate the efficiency at which gp5/trx can initiate DNA synthesis from a primer-template (9). Indeed, we show below that gp5-S-S-trx initiates DNA synthesis with an efficiency similar to that of gp5/trx on a primed poly(dA) DNA template.

To further test the effects of secondary structure on the polymerase activity of gp5-S-S-trx, we performed DNA synthesis assays on linear poly(dA)350-oligo(dT)25, a homopolymeric DNA template lacking secondary structure. gp5-S-S-trx copies poly(dA)350-oligo(dT)25 to a similar extent as gp5/trx, both enzymes incorporating as much as 13,000 pmol of dTMP (Fig. 3C) while replicating all the DNA template in the reaction mixture. Furthermore, gp5-S-S-trx and gp5/trx have nearly identical specific activities on the linear DNA template (61,000 nmol/min/mg versus 62,000 nmol/min/mg). These findings, taken together with those on M13 ssDNA coated with SSB protein, show that gp5-S-S-trx has polymerase activity comparable to that of wild-type gp5/trx provided regions of secondary structure are absent from the DNA template.

The experiments presented in Fig. 3 strongly suggest that the polymerase-thioredoxin complex recycles from one completed M13 DNA template to initiate synthesis on another. Both gp5/trx and gp5-S-S-trx, in the presence of SSB protein copy all of the M13 DNA molecules although there is a 4-fold molar excess of M13 DNA over polymerase (Fig. 3B). Likewise, gp5/S-S-trx and gp5/trx were able to form a stable complex even in the presence of 60 µg of SSB protein per 300-µl volume reaction. (Kd = 5 nM) and dramatically stimulates its activity by increasing the processivity of the polymerase (8, 9). We compared the processivity of gp5-S-S-trx to that of gp5/trx by carrying out DNA synthesis on a M13 ssDNA template annealed with a 5'-32P-labeled 17-nt primer in the absence (A) or presence (B) of E. coli SSB protein as described under “Experimental Procedures.” DNA synthesis reactions (300 µl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM MgCl2, 0.25 mM GTP, dATP, dTTP, and dCTP, 4 nm gp5-S-S-trx or gp5/trx, and 40 nt primed M13 ssDNA. Reaction mixtures were incubated at 37 °C. Aliquots (20 µl) were removed from the reaction at the indicated times and stopped by addition of EDTA to a final concentration of 25 mM. The reaction products were separated by electrophoresis on a 0.6% alkaline agarose gel. The effects of SSB protein were determined in the presence of 60 µg of SSB protein per 300-µl volume reaction.
polymerase complex in order to observe DNA synthesis as the result of a single binding event. As shown in Fig. 4A, the processivity of gp5/trx is thousands of nucleotides within 2 min of DNA synthesis as observed by the sizes of the replication products. However, under the conditions of this assay a number of strong pause sites are observed where the polymerase cannot synthesize DNA efficiently through regions of duplexed DNA (30). In contrast to wild-type gp5/trx, the covalently linked complex is far less processive (Fig. 4A). Even after 30 min of incubation, the products of DNA synthesis are relatively short, with no fully extended products synthesized. These results confirm that the early plateau of DNA polymerase activity by gp5-S-S-trx on M13 ssDNA (Fig. 3A) is due to the complex being halted by secondary structure.

We have shown above that E. coli SSB protein increases the rate and extent of DNA synthesis catalyzed by both gp5-S-S-trx and gp5/trx on primed M13 ssDNA (Table I). In the presence of SSB protein the processivity of both gp5-S-S-trx and gp5/trx are dramatically increased and to the same extent (Fig. 4B). Within 30 s both complexes synthesize thousands of nucleotides to fully replicate the circular M13 DNA template. We conclude that in the presence of SSB protein gp5-S-S-trx has a processivity similar to that of gp5/trx. Thus, these results confirm that the presence of the covalent linkage between the mutant polymerase and thioredoxin in gp5-S-S-trx affects the ability of the complex to polymerize nucleotides through sites of secondary structure.

Exonuclease Activity—gp5/trx has 3′-5′ exonuclease activity that is active on both single- and double-stranded DNA. The 3′-5′ single-stranded DNA exonuclease of gp5/trx has the same high level of activity as gp5 alone, but hydrolysis of duplex DNA is stimulated dramatically by the presence of thioredoxin (9, 13, 14). In view of the differences in processivity observed above for gp5/trx and gp5-S-S-trx on M13 DNA, it was of interest to compare the single- and double-stranded exonuclease activities of both these complexes. Exonuclease activity by the enzymes was determined by measuring hydrolysis of uniformly [3H]labeled single- and double-stranded M13 DNA over time as previously described (37, 39). The exonuclease activity on single-stranded DNA is identical for both gp5/trx and gp5-S-S-trx (data not shown). Furthermore, the exonuclease activity of gp5-S-S-trx on M13 dsDNA was only 2-fold less active than that observed with gp5/trx.

Binding Affinity of gp5(T327C) and trx(C35S)—It has previously been shown that thioredoxin binds tightly to gene 5 protein ($K_d = 5$ nM) and increases the affinity of the polymerase for a primer-template, which in turn leads to a dramatic stimulation of the polymerase activity (9, 10). We quantitatively assayed the ability of gp5-S-S-trx to form active complexes by performing binding studies with procedures modified from those previously described, substituting poly(dA) as a DNA template for calf thymus DNA (Ref. 10 and see “Experimental Procedures”). Using poly(dA)$_{350}$-oligo(dT)$_{25}$ DNA as the primer-template, we measured the amount of [3H]thymidine incorporated by the polymerase as a function of trx or trx(C35S) concentration (Fig. 5A). The observed equilibrium binding constants ($K_{obs}$) were obtained from Scatchard plots of this data (Fig. 5B). Thioredoxin is efficient at forming a complex with gp5 as with gp5(T327C) ($K_{obs} = 59$ nM versus $63$ nM). Furthermore, trx(C35S) and gp5(T327C), the altered proteins used to form gp5-S-S-trx, stimulate polymerase activity, but they bind with a 3-fold lower affinity than the wild-type proteins ($K_{obs} = 225$ nM versus $59$ nM), trx(C35S) and gp5 also bind with a 3-fold lower affinity than the wild-type subunits.

gp5-S-S-trx Recycles—The mechanism by which the gp5/trx complex recycles to another primer-template upon completing

![Figure 5. Binding affinity of gene 5 proteins to thioredoxins.](https://www.jbc.org/content/273/50/23768/F5.large.jpg)
doxin as the polymerase recycles (Fig. 6A). When gp5 and thioredoxin are added to the polymerase reaction in the same concentration as the pre-formed complexes, but as individual components in a 1:1 molar ratio, the rate of dTMP incorporation on poly(dA)350-oligo(dT)25 is only 11% of that for gp5/trx. Similarly, only 4% of polymerase activity is detected when the free gene 5 protein is saturated with a 500- or 1000-fold excess of trx or trx(C35S), respectively. Therefore, we have used these conditions to examine the time course of polymerase activity for the reconstituted complexes (Fig. 6B). For comparative purposes, the amount of free gene 5 protein or gp5/trx was equivalent for each assay. We show that the polymerase activity detected for each reconstituted complex is identical to that for the pre-formed gp5/trx and gp5-S-S-trx complexes.

** Fate of gp5-S-S-trx Blocked by Secondary Structure—** gp5-S-S-trx cannot complete the replication of a primed-M13 DNA template because the covalently linked complex has difficulty polymerizing through sites of secondary structure (Fig. 4A). We have examined the fate of gp5-S-S-trx when it is stalled at secondary structure. When the amount of M13 DNA is doubled in a polymerase assay, the amount of DNA synthesis catalyzed by gp5-S-S-trx is unchanged, whereas wild-type gp5/trx catalyzes the synthesis of twice the amount of DNA (Fig. 7). These data suggest that gp5-S-S-trx cannot rebind to another primer-template after stalling at secondary structure.

To determine the extent to which gp5-S-S-trx dissociates from the M13 DNA after pausing, we initiated synthesis on primed-M13 DNA with gp5-S-S-trx and then added *E. coli* SSB protein after 10 min of DNA synthesis (Fig. 8). Upon addition of SSB protein, inhibition of gp5-S-S-trx by secondary structure is overcome, resulting in a dramatic increase in DNA synthesis. Clearly, gp5-S-S-trx dissociates very slowly from the M13 DNA.

The possibility that trx(C35S) dissociates from gp5(T327C) when gp5-S-S-trx is blocked at secondary structure was examined with DNA synthesis assays with saturating amounts of thioredoxin (Fig. 9). gp5/trx alone is able to synthesize DNA to a level that is nearly equal to the amount of M13 DNA template available in the reaction mixture. Addition of a 500-fold excess of trx stimulates the synthesis by gp5/trx to completely replicate all the available M13 molecules. The reason for this stimulation is clear when we examine gp5-S-S-trx with excess thioredoxin. gp5-S-S-trx alone is able to complete the replication of the M13 DNA after pausing.
polymerase and processivity factor detaching from each other, dissociates from M13 DNA at sites of secondary structure, with the active site cysteines (Cys-32 and Cys-35) are not removed from the reaction at the indicated times and stopped by addition of EDTA to a final concentration of 0.1 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 250 μM each dGTP, dATP, dCTP, and [³H]dTTP (10 cpm/pmol), 14 nM single-stranded, primed M13 DNA, and 4 nM gp5-S-S-trx or gp5/trx. Reaction mixtures were incubated at 37 °C. Aliquots (20 μl) were removed from the reaction at the indicated times and stopped by addition of EDTA to a final concentration of 25 mM. The amount of DNA synthesis for each reaction was determined by the amount of [³H]dTMP incorporated over time. The effect of SSB protein on DNA synthesis by gp5-S-S-trx (closed triangles) was determined by addition of 60 μg of E. coli SSB protein per 300-μl volume reaction at 10 min following the start of the reaction.

**Fig. 8. Rescue of gp5-S-S-trx on M13 ssDNA by E. coli SSB protein.** DNA synthesis by gp5-S-S-trx (open triangles) or gp5/trx (closed circles) was monitored on M13 ssDNA as described under “Experimental Procedures.” DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 250 μM each dGTP, dATP, dCTP, and [³H]dTTP (10 cpm/pmol), 14 nM single-stranded, primed M13 DNA, and 4 nM gp5-S-S-trx or gp5/trx. Reactions were incubated at 37 °C. Aliquots (20 μl) were removed from the reaction at the indicated times and stopped by addition of EDTA to a final concentration of 25 mM. The amount of DNA synthesis for each reaction was determined by the amount of [³H]dTMP incorporated over time. The effect of SSB protein on DNA synthesis by gp5-S-S-trx (closed triangles) was determined by addition of 60 μg of E. coli SSB protein per 300-μl volume reaction at 10 min following the start of the reaction.

Polymerase-Thioredoxin Covalent Complex

**Fig. 9. Effect of excess thioredoxin on DNA synthesis catalyzed by gp5-S-S-trx.** DNA synthesis catalyzed by gp5-S-S-trx (open circles) or gp5/trx (open circles) was monitored on M13 ssDNA as described under “Experimental Procedures.” DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 250 μM each dGTP, dATP, dCTP, and [³H]dTTP (10 cpm/pmol), 14 nM single-stranded, primed M13 DNA, and 4 nM gp5-S-S-trx or gp5/trx. Reaction mixtures were incubated at 37 °C. Aliquots (20 μl) were removed from the reaction at the indicated times and stopped by addition of EDTA to a final concentration of 25 mM. The amount of DNA synthesis for each reaction was determined by the amount of [³H]dTMP incorporated over time. The effect of SSB protein on DNA synthesis by gp5-S-S-trx (closed triangles) was determined by addition of 1000-fold molar excess of trx(C35S) and 500-fold molar excess trx, respectively, over polymerase.

**Discussion**

The DNA polymerase encoded by gene 5 of bacteriophage T7 has low processivity, polymerizing only a few nucleotides before dissociating from a DNA molecule (8). gp5 achieves high processivity upon binding E. coli thioredoxin. Thioredoxin increases the binding affinity of gp5 for a primer-template (9), thereby dramatically stimulating its processivity to thousands of nucleotides incorporated per replication cycle (8). The gp5/trx complex is able to fully replicate a primed M13 ssDNA template (7200 nucleotides) in a single encounter (8). Although thioredoxin binds gp5 with a strong affinity, the fate of thioredoxin during recycling of the polymerase was previously unclear. One possibility had been that thioredoxin remains bound to gp5 as the polymerase recycled. Alternatively, gp5 could have dissociated from thioredoxin after the polymerase was released from a fully replicated DNA molecule. In this latter scenario, gp5 would then rebind free thioredoxin to form another gp5/trx complex to reinitiate DNA synthesis on another DNA template. In the present work we have used an altered gp5/trx complex having thioredoxin covalently attached to gp5 to provide insight into the mechanism of recycling and processivity of wild-type gp5/trx. We find that the gp5/trx complex recycles intact to replicate all the DNA molecules in a reaction in which the DNA is in a molar excess over gp5/trx.

The role of thioredoxin as a processivity factor is unique in that the active site cysteines (Cys-32 and Cys-35) are not re-
required in a catalysis reaction as in the case for thioredoxin in the other reactions in which it participates (22–23). Normally, Cys-32 and Cys-35 are redox-active and participate in various reductive processes. However, altered thioredoxins with Cys-32 and Cys-35 replaced with residues that abolish the enzymatic activity maintain the ability to confer processivity on gp5 (29). Thus, thioredoxin plays only a structural role when bound to gp5. The crystal structure of gp5/trx reveals that Cys-32 forms a hydrogen bond with Thr-327 of gp5 within a flexible loop that extends from the polymerase thumb (17). We used this interaction at the binding surface between gp5 and thioredoxin as a position to engineer a covalent linkage between the two proteins (29). Replacing Thr-327 of gp5 with a cysteine, Cys-32 of thioredoxin formed a stable disulfide linkage with the polymerase after the two proteins associated. We also replaced Cys-35 of thioredoxin with serine so this cysteine would not resolve the interdisulfide linkage of the covalently linked complex gp5-S-S-trx.

gp5-S-S-trx has polymerase activity identical to that of wild-type gp5/trx on linear DNA templates lacking secondary structure. However, the covalently linked complex has markedly lower polymerase activity and processivity on ssM13 DNA since the altered complex has difficulty polymerizing nucleotides through sites of secondary structure. In contrast to gp5/trx, gp5-S-S-trx fails to fully replicate any of the M13 DNA molecules. gp5-S-S-trx becomes stalled at secondary structure and requires the presence of E. coli SSB protein to destabilize the presumably duplex hairpins for further replication. In the presence of SSB protein, gp5-S-S-trx can complete replication of the M13 DNA molecule on which it is stalled as well as recycle to replicate all the DNA templates. In the absence of SSB protein, gp5-S-S-trx remains stalled at sites of secondary structure and slowly dissociates from the DNA, with subsequent detachment of trx(C35S) from gp5(T327C), resulting in a non-processive polymerase. Under the reaction conditions of our assays, trx(C35S) will not rebind with the polymerase once it has dissociated due to its extremely low concentration upon dilution in the reaction mixture. Therefore, stalled gp5-S-S-trx cannot dissociate from pause sites to replicate additional M13 DNA that has been added to the polymerase assay. These findings clearly indicate that thioredoxin must remain bound to the polymerase in order for the polymerase to recycle to finish replication of all the M13 DNA molecules. Only when supplemented with a large excess of trx(C35S) (1000-fold) in the reaction mixture will the dissociated gp5(T327C) form a new gp5-S-S-trx complex. The newly formed complex can then recycle to a new M13 DNA template and reinitiate DNA synthesis through secondary structure. Apparently, the repeated rebinding of new gp5-S-S-trx complexes formed in the presence of excess thioredoxin eventually allows the complex to polymerize through secondary structure (42).

Unlike gp5-S-S-trx, the wild-type gp5/trx complex can circumnavigate a majority of the M13 ssDNA in our DNA replication assays without the presence of SSB protein. However, secondary structure is also a strong hindrance to gp5/trx, and like gp5-S-S-trx, SSB protein is required for gp5/trx to fully replicate all the M13 DNA molecules. It is not surprising to find that the presence of a high molar excess (500-fold) of trx stimulates gp5/trx to copy all the DNA templates. Similar to gp5-S-S-trx, excess thioredoxin rescues free gp5 generated from complexes that have fallen apart at pause sites. Therefore, gp5/trx seems to be committed to replicate through secondary structure, and those stalled complexes that dissociate prematurely from the DNA template do not recycle to a new DNA template because thioredoxin detaches from the polymerase and cannot rebind at the dilute concentration in the reaction mixture. As a negative control, we performed similar experiments but substituted M13 DNA for poly(dA)350-oligo(dT)25 thereby eliminating secondary structure from the assay (data not shown). As expected, there was no difference in the polymerase activity in the presence or absence of excess thioredoxin. This result demonstrates that the increase in activity on M13 DNA observed with excess trx is not due to the gp5/trx preparation having contaminating gp5.

Although our reactions show that gp5 recycles with thioredoxin as a complex on ssDNA templates they do not address the fate of thioredoxin as the polymerase recycles at a replication fork. A replisome consisting of T7 gene 5 protein/thioredoxin, T7 gene 4 helicase-primase, and gene 2.5 SSB protein can mediate coordinated DNA synthesis in vitro (15, 16). In this coordinated system, leading and lagging strand synthesis proceed at identical rates, a replication loop of lagging strand DNA is present, and both the leading and lagging strand DNA polymerases are processive. The lagging strand polymerase is processive in the sense that it is responsible for synthesis of all of the Okazaki fragments on a given DNA molecule. At the completion of an Okazaki fragment, the lagging strand polymerase must dissociate from a completed Okazaki fragment and initiate synthesis at a new primer without leaving the replisome. It is reasonable to postulate from our results that the polymerase remains complexed with thioredoxin as the lagging strand polymerase recycles from one Okazaki fragment to the next. Just as gp5 becomes non-processive when trx dissociates during replication of M13 ssDNA templates, one can envisage a similar result for the lagging strand polymerase.

The ability of gp5/trx to synthesize through sites of secondary structure is related to its ability to strand displacement (42). In general, gp5/trx is poor at strand displacement synthesis as compared with other DNA polymerases, such as δ29 DNA polymerase. The δ29 DNA polymerase has the ability to replicate long stretches of duplex DNA without the requirement of a helicase because it is very efficient at strand displacement.
allow polymerization through the hairpin by strand displacement synthesis. It is possible that the disulfide linkage between gp6(T232C) and trx(C55S) leads to restricted conformation that impedes the ability of the complex to make the necessary conformational changes for nucleotide turnover and strand displacement synthesis.

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REFERENCES


