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A Covalent Linkage between the Gene 5 DNA Polymerase of Bacteriophage T7 and *Escherichia coli* Thioredoxin, the Processivity Factor

FATE OF THIOREDOXIN DURING DNA SYNTHESIS*

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Gene 5 protein (gp5) of bacteriophage T7 is a non-processive DNA polymerase, which acquires high processivity by binding to *Escherichia coli* thioredoxin. The gene 5 protein-thioredoxin complex (gp5/trx) polymerizes thousands of nucleotides before dissociating from a primer-template. We have engineered a disulfide linkage between the gene 5 protein and thioredoxin within the binding surface of the two proteins. The polymerase activity of the covalently linked complex (gp5-S-S-trx) is similar to that of gp5/trx on poly(dA)/oligo(dT). However, gp5-S-S-trx has only one third the polymerase activity of gp5/trx on single-stranded M13 DNA. gp5-S-S-trx has difficulty polymerizing nucleotides through sites of secondary structure on M13 DNA and stalls at these sites, resulting in lower processivity. However, gp5-S-S-trx has an identical processivity and rate of elongation when *E. coli* single-stranded DNA-binding protein (SSB protein) is used to remove secondary structure from M13 DNA. Upon completing synthesis on a DNA template lacking secondary structure, both complexes recycle intact, without dissociation of the processivity factor, to initiate synthesis on a new DNA template. However, a complex stalled at secondary structure becomes unstable, and both subunits dissociate from each other as the polymerase prematurely releases from M13 DNA.

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 encirles the DNA to tether the polymerase to a primed DNA template (4–6). For example, *Escherichia coli* DNA polymerase III must have its processivity factor, the β-clamp, pre-assembled on the primer-template before the polymerase can productively bind to the DNA to initiate DNA synthesis. In contrast, several viruses utilize a different mechanism to achieve high processivity. For example, the DNA polymerase of bacteriophage T7 adopts the host protein *E. coli* thioredoxin (trx)[1] 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 (K_d, ~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 the thumb 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-

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[1] The abbreviations used are: trx, thioredoxin; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; nt, nucleotide; SSB, single-stranded DNA-binding; wt, wild type.

sive mode the thumb and bound thioredoxin is postulated to swing down onto the duplex portion of the primer-template to prevent the DNA from dissociation prior to the next polymerization cycle. Suppressor analysis of a genetically altered thioredoxin supports this scenario (21). Amino acids in gp5 that restore the ability of the altered thioredoxin to confer processivity on the polymerase reside within the thioredoxin binding segment while another is located within the exonuclease domain. The latter site is interesting since it raises the possibility that the extended loop of the thioredoxin binding segment might swing down and dock on the lip of the crevice located within the exonuclease domain, thus encircling gp5/trx around the DNA within a structure similar to a sliding clamp. Alternatively, thioredoxin could be increasing the electrostatic interactions between the polymerase and the DNA template.

At a replication fork, gp5/trx interacts with the T7 gene 4 helicase-primase (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. 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.[2] This interaction effectively decreases the polarity of Thr-327 within the hydrophobic 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

Bacterial Strains and DNA—Bacterial strain BL21(DE3), used to express wild-type thioredoxin, was purchased from Invitrogen. *E. coli* A307 (HrfC, Δ*trxA307*) was a gift from Stan Tabor (Harvard Medical School). *E. coli* A307(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 λDE3 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 was purified using a Lambda Maxi kit purchased from Qiagen, Inc. Poly(dA)$_{350}$-oligo(dT)$_{25}$ and oligonucleotide primers for M13 DNA were obtained from Midland Certified Reagent Co. Plasmids pGP5-3, pTrx-3, and pGP1-2, vectors having wild-type T7 gene 5, *E. coli trx*A, and T7 gene 1, respectively, were gifts from Stan Tabor. Plasmid pT7-7, the parent vector of pGP5-3 and pTrx(C35S)-1, was a gift from Stan Tabor. Plasmid pET-24a, the parent vector of pTrxA and pTrx(C35S)-2, was purchased from Novagen.

Mutagenesis of T7 Gene 5—Plasmid pGP5(T327C) was constructed by mutagenesis of T7 gene 5 within pGP5-3 using an "overlap extension" method (34). The mutagenesis required three separate PCR reactions using using *PfuTurbo* DNA polymerase (Stratagene). The first PCR reaction used the primers DJ4 (5′-GAAGGGTTAAACACAACATGTTCAACTGGGCAGTAAGGAGCACCAGCAACGTACT-3′) and BCMP97 (5′-GCATTGACCAAACTGGCAAAG-3′) to generate a 5′ fragment of T7 gene 5 that contains a codon that corresponds to a Thr-327 to Cys alteration. The altered codon of primer DJ4 is underlined. A 3′ fragment containing the same codon was generated in a second PCR reaction using primers DJ3 (5′-AGTACGTTGCTGGTGCTCCTTACTGCCCAGTTGAACATGTTGTGTTTAACCC-3′) and JH8 (5′-GCGAGCCATGAAGTGAGCC-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 purified on an agarose gel, digested with *Sty*I and *Mfe*I, and ligated into corresponding sites on plasmid pGP5-3 to create pGP5(T327C). The desired clone was confirmed by DNA sequencing.

Mutagenesis of E. coli trxA—Plasmid pTrx(C35S) was constructed by mutagenesis of *E. coli trx*A of plasmid pTrx-3 using a "Megaprimer" method (35). Plasmid pTrx-3 contains a copy of wild-type *E. coli trx*A. The mutagenesis required two separate PCR reactions using *PfuTurbo* DNA polymerase (Stratagene). The first PCR reaction used primer A (5′-GAGTGGTGCGGTCCGTCCAAAATGATCGCCCCGATT-3′) and primer B (5′-GCTTCTAAGCTTCCCTTACGCCAGGTT-3′) to generate a 5′ fragment of the *trx*A 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′-GTTGGTAGCGGCCATATGAGCGATAAAATTATTCAC-3′) to generate a full-length copy of the *trx*A gene with the desired mutation. The final PCR product

[2] D. Johnson, S. Tabor, T. Ellenberger, and C. C. Richardson, unpublished results.

was purified on an agarose gel, digested with *Nde*I and *Hin*dIII, and ligated into corresponding sites on plasmid pT7-7 to create pTrx (C35S)-1, the plasmid used to express trx(C35S) in the presence of ampicillin for purification. The restricted fragment containing the mutated *trx*A 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 *trx*A of plasmid pTrx-3 using primer A and primer C, followed by digestion with *Nde*I and *Hin*dIII, and then ligation into corresponding sites on plasmid pET-24a. The desired clones were confirmed by DNA sequencing.

Other Materials—*PfuTurbo* DNA polymerase or *AmpliTaq* DNA polymerase (Applied Biosystems) were used for standard PCR reactions. Unlabeled nucleotides (HPLC grade) and [*methyl*-^{3}H]TTP (3000 Ci/mmol) were obtained from Amersham Biosciences. Restriction enzymes *Nde*I, *Hin*dIII, *Sty*I, and *Mfe*I were purchased from New England Biolabs. T4 DNA ligase, T4 polynucleotide kinase, and ThermoSequenase were from Amersham Biosciences. IPTG, DTT, kanamycin, and ampicillin were obtained from American Bioanalytical. DEAE-cellulose (DE52), phosphocellulose (P11), and DE81 filter papers were obtained from Whatman Paper Ltd. Sephadex G-50, HiTrap heparin columns (5-ml bed volume), HiTrap Q Sepharose HP columns (5-ml bed volume), and agarose-HE were from Amersham Biosciences. Ceramic hydroxyapatite columns (5-ml Econo-*Pac*CHT-II cartridges) and bovine serum albumin were from Bio-Rad.

Methods

Plating Efficiencies—Plating efficiencies of T7Δ5 phage were measured on *E. coli* A307(ΔtrxA) harboring either plasmid pT7-7, pGP5-3, pGP5(T327C), pET-24a, pTrxA, pTrx(C35S)-2, or a combination of two of these plasmids (Table II). Cells having plasmids pT7-7, pGP5-3, 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 T7Δ5 phage (100 μl) were mixed with a 100-μl plating culture and 3 ml of top agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% agar, pH 7.0) that was preincubated at 48 °C. Mixtures were plated on TB plates at room temperature (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7.0) having appropriate antibiotics. The plates were incubated at 37 °C for 5 h. The efficiency of plating was determined by dividing the number of plaque-forming units by the amount of plaque-forming units observed for cells having pTrxA and pGP5-3 and represents an average of at least two experiments.

Protein Purification—gp5/trx, gene 5 protein, and gp5(T327C) were overexpressed in *E. coli* A307(DE3) using plasmids pGP5-3/pTrxA, pGP5-3, and pGP5(T327C), respectively and then purified using procedures described previously (3). However, hydroxyapatite chromatography was omitted from the purification procedures of gene 5 protein and gp5(T327C). Wild-type thioredoxin was overexpressed in *E. coli* BL21(DE3) using plasmid pTrxA and purified as described (20). trx(C35S) is toxic to growth of *E. coli* BL21(DE3) (data not shown), so this protein was overexpressed in *E. coli* A307 using a heat shock system as described (36). The heat shock system is a two-plasmid system that maintains toxic proteins under tight transcriptional control. One plasmid, pGP1-2, has the gene for T7 RNA polymerase controlled by the λ P_L promoter, the gene for the temperature-sensitive λ repressor cI857, and the gene for kanamycin resistance. The second plasmid is pTrx(C35S)-1, which has the gene for trx(C35S) under control of a T7 promoter and carrying the gene for ampicillin resistance. The λ repressor cI857 tightly represses expression of T7 RNA polymerase at 25 °C, but exclusive expression of trx(C35S) is achieved after heat induction of T7 RNA polymerase at 42 °C. Thus, *E. coli* A307 was transformed with both plasmids pGP1-2 and pTrx(C35S)-1 in the presence of kanamycin and ampicillin, and then initially grown at 25 °C until an OD_{600} of 1. The cells were heat-induced at 42 °C for 1 h followed by protein synthesis for 3 h at 37 °C. trx(C35S) was then purified from cells using procedures previously described for wild-type thioredoxin (36).

The covalently linked gp5-S-S-trx was formed by mixing 20 μM purified gp5(T327C) and 150 μM trx(C35S) overnight at 0 °C in 4 ml of buffer containing 40 mM potassium phosphate (pH 7.4), 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol. gp5-S-S-trx was purified from both free gp5(T327C) and trx(C35S) on a HiTrap heparin column (5-ml bed volume) at 4 °C after diluting the mixture 10-fold with Buffer H (50 mM 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 trx(C35S). gp5-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, gp5-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 gp5-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-Cl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM DTT, 50 mM NaCl, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol). Reactions also had 14 nM single-stranded M13 mp8 DNA primed with a 17-nt oligonucleotide (−40 primer) and 4 nM of either gp5/trx or gp5-S-S-trx. The polymerase assays 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 [^{3}H]dTMP incorporated was measured by liquid scintillation counting. Assays to study the effect of *E. coli* SSB protein on polymerase activity contained 60 μg of SSB protein per 300 μl of reaction mixture. Reactions 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$(dT)_{25}$ 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$(dT)_{25}$ were incubated at 25 °C.

M13 DNA (100 nM) was primed for polymerase assays by annealing with a 17-nt primer (−40 primer) in 50 mM Tris-Cl (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 0 °C for 30 min.

Exonuclease Activity—The 3′ to 5′ single- and double-stranded DNA exonuclease activity of gp5/trx and gp5-S-S-trx were assayed using procedures modified from those previously described (37, 39). Uniformly ^{3}H-labeled double-stranded M13 DNA was prepared by annealing a 17-nt primer (−40 primer) to M13 mp8 DNA. The primer was partially extended by 8 nM gp5/trx in a DNA polymerase reaction containing 10 mM $MgCl_2$, 0.1 mM DTT, 20 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol). The reaction was 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. Radiolabeled single-stranded M13 DNA was prepared by alkali denaturation of ^{3}H-labeled double-stranded M13 DNA by treatment with 50 mM NaOH at room temperature for 15 min followed by neutralization with HCl.

The double-stranded DNA exonuclease assays (200 μl) contained 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM DTT, 50 mM NaCl, 16 nM gp5/trx or gp5-S-S-trx, and 8 nM ^{3}H-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 [^{3}H]DNA remaining was measured by liquid scintillation counting 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 8 nM gp5/trx or gp5-S-S-trx and ~8 nM ^{3}H-labeled, single-stranded M13 mp8 DNA. Assays to study the effect of SSB protein on polymerase activity contained 40 μg of SSB protein per 200-μl volume reaction mixture.

Processivity Assays—Processivity assays were carried out on a single-stranded M13 DNA primed with a 5′ ^{32}P-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 (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dTTP, and dCTP, 4 nM gp5-S-S-trx or gp5/trx, and 40 nM single-stranded, primed M13 DNA. The M13 DNA was in 10-fold molar excess over polymerase. Reactions were incubated at 37 °C, and 20 μl aliquots were removed at the indicated

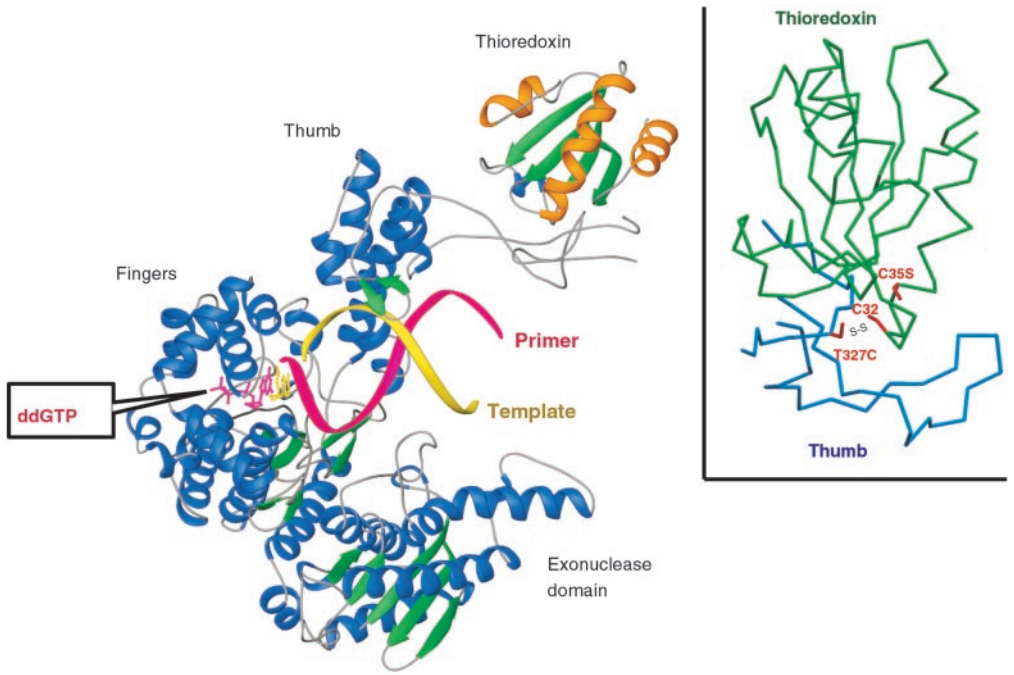

FIG. 1. **Structure of gp5/trx.** The crystal structure of gene 5 protein complexed with thioredoxin, primer-template, and incoming nucleotide at 2.2 Å resolution (17). *Inset*, the gp5 thumb-thioredoxin interface. Thr-327 of the gene 5 protein was substituted for cysteine to create a disulfide bond with Cys-32 of thioredoxin. Thioredoxin Cys-35 was substituted for serine to avoid a potential attack of the sulfhydryl of Cys-35 on the disulfide linkage formed between the two mutant subunits of gp5/trx.

times. Reactions were 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 (33). The gels were dried and exposed to a phosphorus imaging plate followed by scanning with a Fuji BAS 1000 bio-imaging analyzer. The effects of SSB protein were determined in the presence of 60 μg of SSB protein per 300-μl reaction.

Primers for processivity assays were labeled at the 5′-terminus using polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. Radiolabeling reactions were incubated at 37 °C for 60 min followed by heating at 75 °C for 5 min. M13 DNA (100 nM) was primed for processivity assays by annealing with a 17-nt primer (−40 primer) in 50 mM Tris-Cl (pH 7.5) and 50 mM NaCl. Annealing reactions were at 75 °C for 5 min followed by 30 min at room temperature. Primed-M13 DNA for processivity assays was purified using S400 spin columns (Amersham Biosciences).

Reconstitution of Gene 5 Protein-Thioredoxin—The gene 5 protein-thioredoxin complex was reconstituted using purified gene 5 protein and increasing amounts of purified thioredoxin. The reconstituted complex was used in DNA polymerase reactions (20 μl) containing 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol), 200 nM poly(dA)$_{350}$-oligo(dT)$_{25}$, and 4 nM gene 5 protein. The reactions were incubated at 25 °C. Increasing amounts of thioredoxin (0, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000, 2000, 4000, 8000 nM) were added to each reaction. DNA synthesis was measured as described for the polymerase assay. The data were used to generate Scatchard plots for each gene 5 protein-thioredoxin complex. The observed equilibrium constant (K_{obs}) for each complex was determined as the negative slope of the corresponding plot. Reconstitution assays shown in Fig. 6 had 4 nM gene 5 protein in each reaction mixture. The reconstituted complex was formed with thioredoxin in a 1:1 molar ratio or 1000-fold molar excess compared with gene 5 protein.

RESULTS

A Mixed Disulfide between Gene 5 Protein and Thioredoxin—Thioredoxin binds to a flexible loop located between helices H and H1 of the thumb of gene 5 protein (Fig. 1). Although the two active site cysteines of thioredoxin are located at the surface of the protein (24, 25), neither residue forms a disulfide bond with gene 5 protein (28). The crystal structure of gp5/trx reveals that Cys-32 of thioredoxin participates in a hydrogen bond with Thr-327 of gene 5 protein (17). We have used this contact point between thioredoxin and gene 5 protein as a basis 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." *E. coli* strains used to overexpress gp5(T327C) and trx(C35S) lacked *trx*A so that wild-type thioredoxin would not contaminate the protein preparations. gp5(T327C) (20 μM) 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 °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

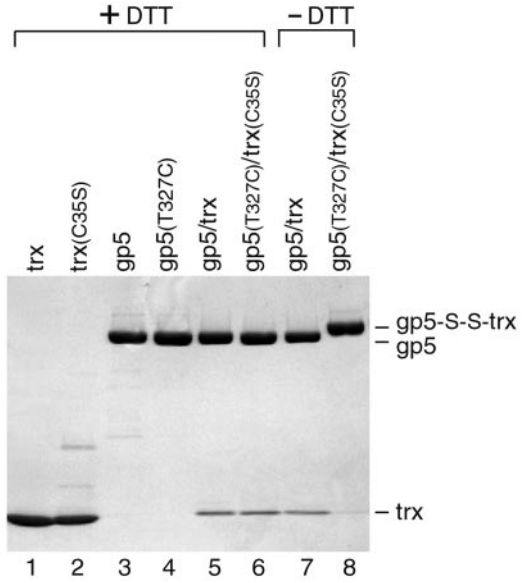

FIG. 2. **Gel analysis of gene 5 protein-thioredoxin.** Gel electrophoresis was on a 4–20% gradient SDS-polyacrylamide gel. Samples in *lanes 1–6* were treated with loading buffer to give final concentrations of 25 mM Tris-Cl (pH 6.8), 50 mM DTT, 1% SDS, 0.05% bromphenol blue, and 5% glycerol. Samples in *lanes 7* and *8* initially contained 0.5 mM DTT and were treated with 25 mM NEM and loading buffer lacking DTT to create non-reducing conditions during gel electrophoresis. *Lane 1* contains purified wild-type thioredoxin. *Lane 2* contains purified trx(C35S). *Lane 3* contains purified wild-type gene 5 protein. *Lane 4* contains purified gp5(T327C). *Lane 5* contains purified gp5/trx. *Lane 6* contains purified gp5(T327C)/trx(C35S) complex. *Lane 7* contains purified gp5/trx under non-reducing conditions. *Lane 8* contains purified gp5-S-S-trx under non-reducing conditions.

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. 3*A*, 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-fold 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

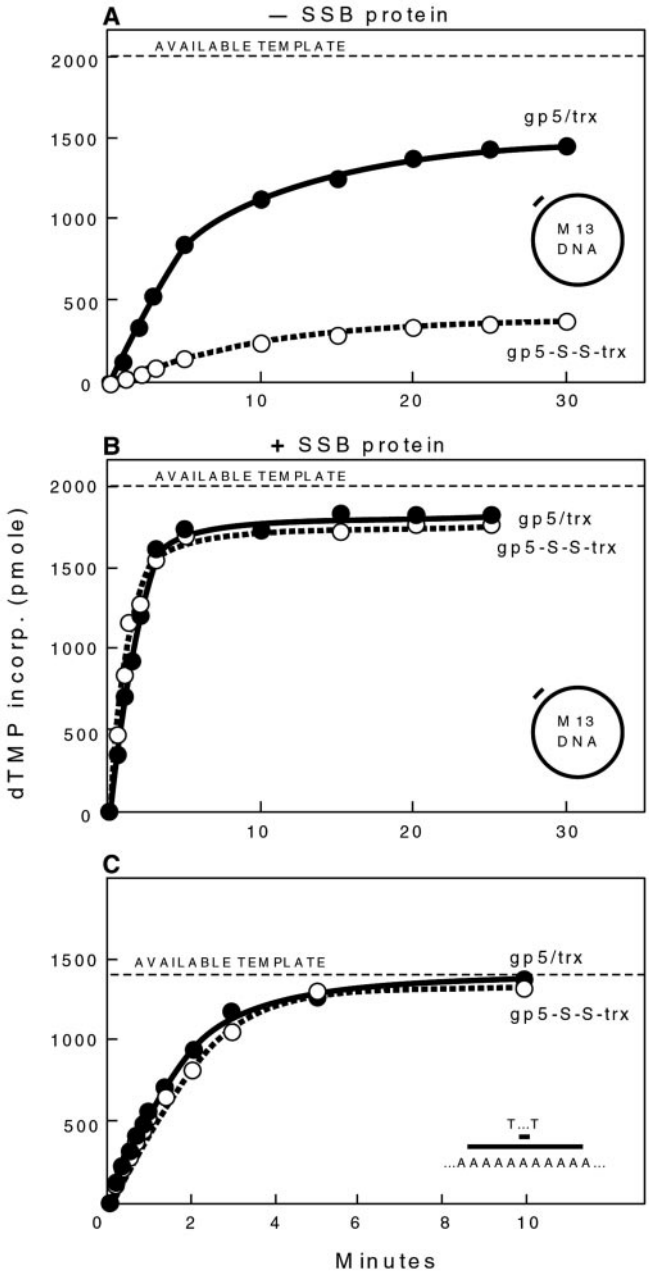

FIG. 3. **Polymerase activity of gp5-S-S-trx and gp5/trx on M13 ssDNA and poly(dA)$_{350}$-oligo(dT)$_{25}$.** The rate of DNA synthesis by gp5-S-S-trx (*open circles*) or gp5/trx (*closed circles*) was determined by the amount of [^{3}H]dTMP incorporated over time as described under "Experimental Procedures." *A*, DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol), 14 nM primed M13 ssDNA, 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. *B*, the effect of *E. coli* SSB protein on polymerase activity on M13 ssDNA was determined by adding 60 μg of SSB protein per 300-μl volume of reaction. *C*, reactions for polymerase activity on poly(dA)$_{350}$-oligo(dT)$_{25}$ were similar for those having M13 ssDNA except poly(dA)$_{350}$-oligo(dT)$_{25}$ was added at a concentration of 200 nM, resulting in a 50-fold molar excess of DNA molecules over polymerase. Additionally, reactions involving poly(dA)$_{350}$-oligo(dT)$_{25}$ were incubated at 25 °C.

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).

TABLE I
Polymerase activity of gp5/trx and gp5-S-S-trx

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 [^{3}H]TMP over time in the presence or absence of *E. coli* single-stranded DNA binding protein.

Gene 5 protein/thioredoxin	Specific activity[a]	
	M13 ssDNA	poly(dA)$_{350}$-oligo(dT)$_{25}$
gp5/trx	22,000	62,000
gp5-S-S-trx	4000	61,000
gp5/trx + SSB protein	78,000	
gp5-S-S-trx + SSB protein	81,000	

[a] 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. 3*B*). 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. 3*B* 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. 3*C*) 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. 3*B*). Likewise, given an even greater fold excess of poly(dA) over polymerase (50-fold), all of the template molecules are copied by either complex (Fig. 3*C*). In a later section we show that thioredoxin does not dissociate from gene 5 protein as the complex recycles. We specifically address this point by showing that if gp5/trx dissociated at the protein concentrations used in these experiments, gp5 and trx cannot re-form a stable complex in the time frame of the reaction. Therefore, thioredoxin must remain bound to gp5 during recycling in order to stimulate gp5 to catalyze the synthesis of all the excess molecules of M13 DNA.

Processivity of gp5-S-S-trx—The gene 5 protein of bacteriophage T7 is a DNA polymerase of extremely low processivity, incorporating only a few nucleotides each binding event of the polymerase (8). Thioredoxin binds tightly to the polymerase

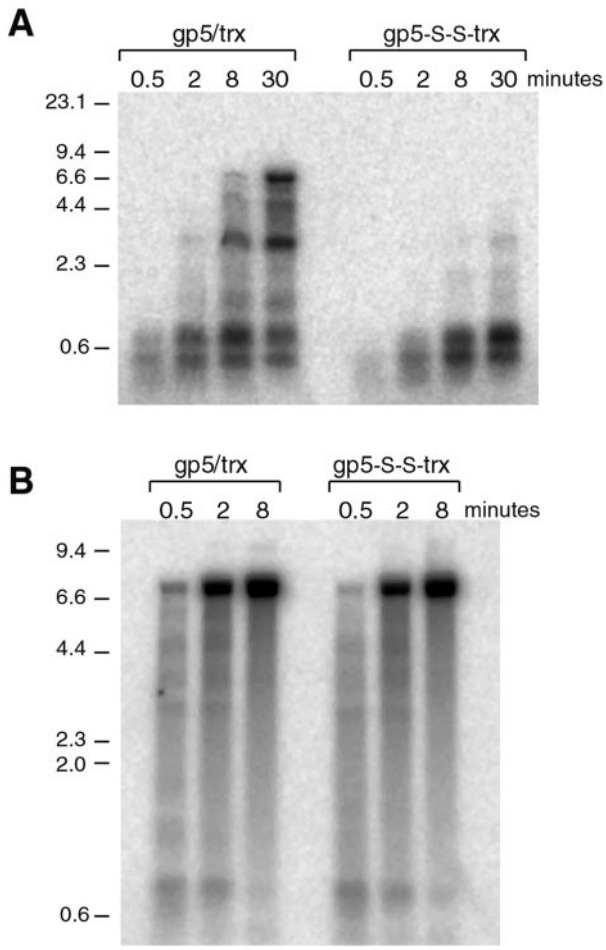

FIG. 4. **Processivity of gp5-S-S-trx and gp5/trx on M13 ssDNA.** Processivity assays were carried out on a M13 ssDNA primed with a 5′ ^{32}P-labeled 17-nt primer in the absence (*A*) or presence (*B*) of *E. coli* SSB protein as described under "Experimental Procedures." DNA synthesis reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 0.25 mM dGTP, dATP, dTTP, and dCTP, 4 nM gp5-S-S-trx or gp5/trx, and 40 nM 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.

(K_d, ~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′ ^{32}P-labeled primer as previously reported (8). Thus, the products of DNA synthesis could be observed over time on an agarose gel. Reactions were performed under conditions in which the primer-template was in 10-fold molar excess over the

polymerase complex in order to observe DNA synthesis as the result of a single binding event. As shown in Fig. 4*A*, 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. 4*A*). 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. 3*A*) 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. 4*B*). 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 ^{3}H-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 [^{3}H]thymidine incorporated by the polymerase as a function of trx or trx(C35S) concentration (Fig. 5*A*). The observed equilibrium binding constants (K_{obs}) were obtained from Scatchard plots of this data (Fig. 5*B*). 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

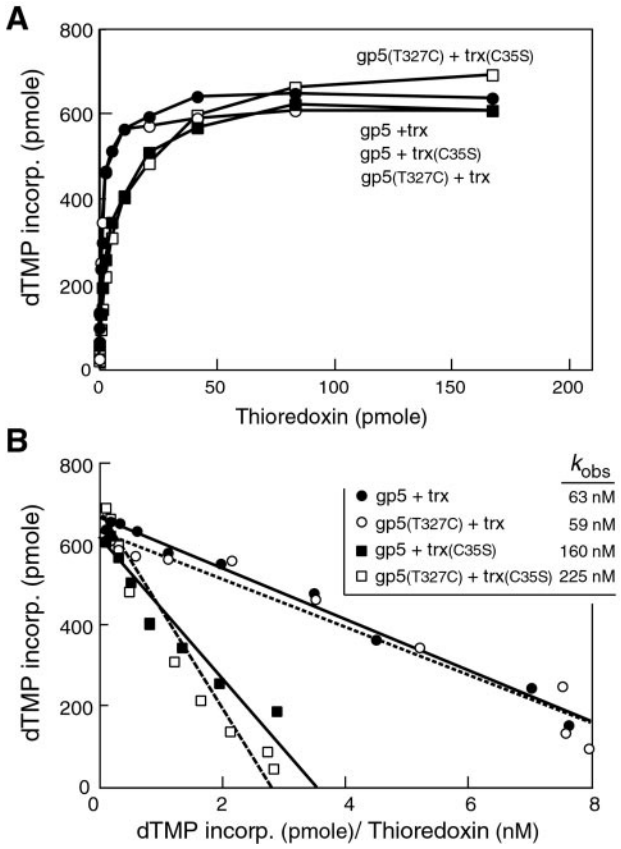

FIG. 5. **Binding affinity of gene 5 proteins to thioredoxins.** *A*, gp5-thioredoxin complexes were formed using purified gp5 or gp5(T327C) proteins mixed with increasing amounts of trx or trx(C35S). The activity of the complexes was measured in a DNA polymerase activity assay as described under "Experimental Procedures." Gene 5 protein and thioredoxin combinations were gp5/trx (*closed circles*), gp5(T327C)/trx (*open circles*), gp5/trx(C35S) (*closed squares*), gp5(T327C)/trx(C35S) (*open squares*). DNA polymerase reactions (20 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol), 200 nM poly(dA)$_{350}$-oligo(dT)$_{25}$ and 4 nM gp5 or gp5(T327C) incubated at 25 °C. Increasing amounts of trx or trx(C35S) were added to each reaction as indicated in the figure. The amount of DNA synthesis for each reaction was determined by the amount of [^{3}H]dTMP incorporated over 1 min. *B*, the data in *A* were used to generate Scatchard plots for each gene 5 protein/thioredoxin complex. The observed equilibrium constant (K_{obs}) for each complex was determined as the negative slope of the corresponding plot.

synthesis on an initial primer-template remains unanswered. The availability of a covalently linked polymerase-thioredoxin complex in which the processivity factor cannot dissociate has allowed us to address this question. It is reasonable to postulate that thioredoxin remains associated with the polymerase, and both subunits recycle together as a tightly bound complex. Alternatively, the polymerase may shed its processivity factor as it dissociates from a completed DNA template to bind another thioredoxin molecule prior to initiating synthesis on a new DNA template.

Results from experiments in which both gp5/trx and gp5-S-S-trx were used to perform DNA synthesis on an excess of poly(dA)$_{350}$- oligo(dT)$_{25}$ suggest that both polymerase/thioredoxin complexes recycle (Fig. 6*A*). Although there is a large molar excess of primer-template over the polymerase (4 pmol *versus* 0.08 pmol) both gp5/trx and gp5-S-S-trx replicate all available template, necessitating multiple rounds of recycling. Although we can conclude from these results that both complexes recycle, it is unclear whether thioredoxin remains intact with the polymerase. To examine this point in greater detail, we used reconstitution assays to examine the fate of thiore-

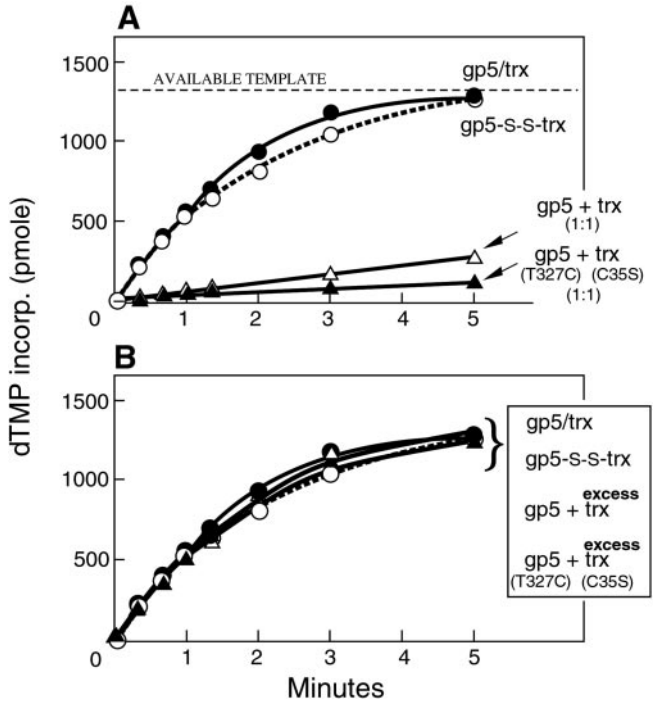

FIG. 6. **Reconstitution assays with gp5/trx and gp5-S-S-trx.** *A*, DNA polymerase assays were performed with purified gp5 and trx (*open triangles*) or purified gp5(T327C) and trx(C35S) (*closed triangles*) at a 1:1 molar ratio with procedures described under "Experimental Procedures." These assays were compared with a plot of DNA polymerase activity for gp5/trx (*closed circles*) and gp5-S-S-trx (*open circles*). DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol), 200 nM poly(dA)$_{350}$-oligo(dT)$_{25}$ and 4 nM gp5, gp5(T327C), gp5/trx, or gp5-S-S-trx. Reaction mixtures were incubated at 25 °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 [^{3}H]dTMP incorporated over time. *B*, DNA polymerase assays were performed with similar procedures as above using 4 nM gp5/trx (*closed circles*), gp5-S-S-trx (*open triangles*), purified gp5 and 500-fold molar excess trx (*open triangles*), or purified gp5(T327C) and 1000-fold molar excess trx(C35S) (*closed triangles*).

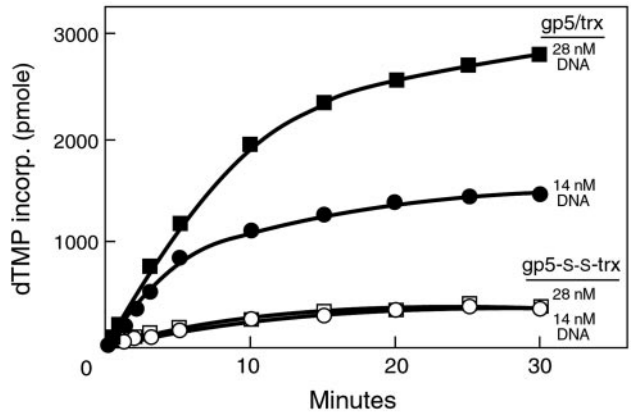

FIG. 7. **Ability of gp5/trx to recycle on M13 ssDNA.** DNA synthesis by gp5-S-S-trx (*open circles*) or gp5/trx (*closed circles*) was monitored on M13 ssDNA as described under "Experimental Procedures." The effect of doubling the amount of M13 ssDNA is shown for both gp5-S-S-trx (*open squares*) and gp5/trx (*closed squares*). DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (10 cpm/pmol), 14 nM or 28 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 [^{3}H]dTMP incorporated over time.

doxin as the polymerase recycles (Fig. 6*A*). 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 trx(C35S) and gp5(T327C) are added to the reaction as separate components. These data clearly demonstrate that under these reaction conditions the polymerase cannot efficiently form another complex if it had dissociated from thioredoxin after completing DNA synthesis on a primer-template. Furthermore, these results are consistent with dissociation constants for gp5/trx and gp5/trx(C35S) complexes (K_{obs} = 59 nM and 160 nM, respectively; Fig. 5*B*), which suggest that the polymerase and thioredoxin, at the concentrations within our experiments (4 nM polymerase and thioredoxin), cannot reform stable complexes upon dissociation. Thus, we conclude that the complexes recycle intact. Furthermore, gp5-S-S-trx replicates poly(dA)$_{350}$- oligo(dT)$_{25}$ DNA at the same rate as the wild-type complex (Table I), suggesting the disulfide linkage of gp5-S-S-trx does not affect the efficiency of this complex to recycle as compared with gp5/trx.

As a control to the above experiments we demonstrate in Fig. 6*B* that the subunits, when added separately to the polymerase assay, have the ability to reconstitute the activity of their respective pre-formed complexes, but only when the processivity factor is present in high molar excess. We have previously shown (Fig. 5*A*) that maximal polymerase activity is achieved on a poly(dA)$_{350}$- oligo(dT)$_{25}$ by either a gp5 or gp5(T327C) 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. 6*B*). For comparative purposes, the amount of free gene 5 protein or gp5/trx was equivalent for each assay. We show that the polymerase activity plotted 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. 4*A*). 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

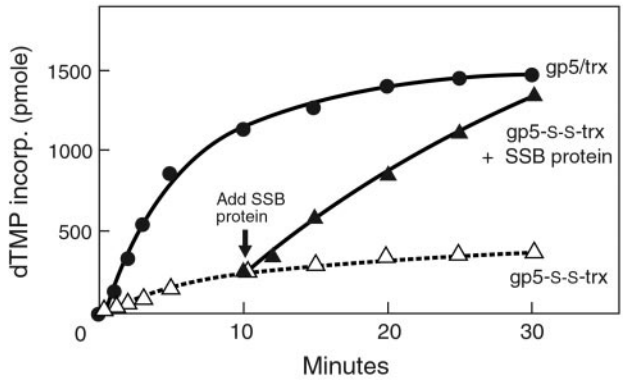

FIG. 8. **Rescue of gp5-S-S-trx on M13 ssDNA by *E. coli* SSB protein.** DNA synthesis by gp5-S-S-trx (*open triangles*) or gp5/trx (*closed circles*) was monitored on M13 ssDNA as described under "Experimental Procedures." DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (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 [^{3}H]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.

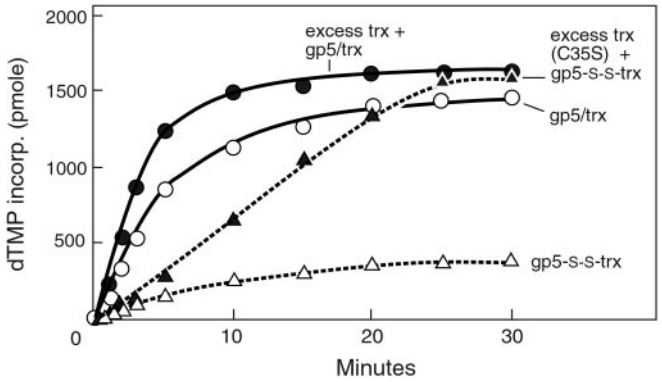

FIG. 9. **Effect of excess thioredoxin on DNA synthesis catalyzed by gp5-S-S-trx.** DNA synthesis catalyzed by gp5-S-S-trx (*open triangles*) or gp5/trx (*open circles*) was monitored on M13 ssDNA as described under "Experimental Procedures." DNA polymerase reactions (300 μl) contained 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 250 μM each dGTP, dATP, dCTP, and [^{3}H]TTP (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 [^{3}H]dTMP incorporated over time. The effect of trx(C35S) on DNA synthesis by gp5-S-S-trx (*closed triangles*) or trx on DNA synthesis by gp5/trx (*closed circles*) was determined by addition of 1000-fold molar excess of trx(C35S) and 500-fold molar excess trx, respectively, over polymerase.

of only a fraction of all the primed-M13 DNA, because it becomes stalled at secondary structure. However, when a molar excess of trx(C35S) is present, the polymerase activity does not plateau early but increases until it reaches a similar level to that achieved by gp5/trx. We propose gp5-S-S-trx slowly dissociates from M13 DNA at sites of secondary structure, with the polymerase and processivity factor detaching from each other, resulting in a non-processive enzyme. At the concentration of proteins used in these assays, the components of gp5-S-S-trx are unable to bind efficiently to form another complex once they have dissociated (Fig. 6*A*). However, in the presence of excess trx(C35S), the free gp5(T327C) binds another processivity factor to form a functional complex that can re-associate with M13 DNA to continue processive DNA synthesis.

Effects of gp5(T327C) and trx(C35S) on Phage Growth—In view of the difference in processivity of gp5-S-S-trx compared with gp5/trx, we were curious as to the ability of the two mutant subunits gp5(T327C) and trx(C35S) to form functional complexes *in vivo*. Therefore, we performed complementation assays to study the ability of gp5(T327C) and trx(C35S) to support T7 phage growth on *E. coli*. The effects of the mutations in the structural genes for gp5 and trx were determined by measuring the ability of the altered proteins to complement the growth of T7 phage lacking gene 5 (T7Δ5 phage) on *E. coli* A307 (ΔtrxA), a bacterium lacking thioredoxin. Mutant forms of gene 5 protein and thioredoxin were expressed *in vivo* from plasmids encoding an ampicillin and kanamycin resistance, respectively, so that both could be maintained inside *E. coli* simultaneously.

As shown by the plating efficiencies in Table II, T7Δ5 phage cannot grow when either wild-type gp5 or gp5(T327C) are expressed alone from a plasmid in host *E. coli* A307 lacking thioredoxin. Similarly, expression of trx or trx(C35S) in the absence of exogenous gene 5 protein cannot support growth of T7Δ5 phage. As expected, T7Δ5 phage produce viable progeny when both wild-type gp5 and trx are present. Interestingly, T7Δ5 phage also grow on *E. coli* A307 expressing gp5 and trx(C35S), demonstrating that trx(C35S) can substitute for wild-type thioredoxin as a processivity factor for gene 5 protein. These results are in agreement with those previously reported by Huber *et al.* (29) that show that thioredoxin with substitutions at the active site cysteines is able to support T7 phage growth.

Simultaneous expression of gp5(T327C) and trx(C35S) results in a 10-fold lower plating efficiency of T7Δ5 phage on *E. coli* A307 as compared with wild-type gp5 and trx. Similarly, the presence of gp5(T327C) and trx together yield viable phage at a significantly lower efficiency. These results suggest that a complex formed *in vivo* by gp5(T327C) binding with either trx(C35S) or wild-type trx does not have a normally functioning polymerase, presumably reflecting the same properties of the gpr-S-S-trx complex we have examined *in vitro*.

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 senerio, 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-

TABLE II
Ability of gene 5 and trxA mutants to complement phage growth

Plasmid	Mutation	Efficiency of plating[a] T7Δ5
pET-24a	No trxA	$<10^{-6}$
pTrx	Wt trxA	$<10^{-6}$
pTrx(C35S)	trx(C35S)	$<10^{-6}$
pT7-7	No gene 5	$<10^{-6}$
pGP5-3	Wt gene 5	$<10^{-6}$
pGP5(T327C)	gp5 (T327C)	$<10^{-6}$
pT7-7/pET-24a	No gene 5/no trxA	$<10^{-6}$
pGP5-3/pTrx	Wt gene 5/wt trxA	1.0
pGP5-3/pTrx(C35S)	Wt gene 5/trx(C35S)	0.9
pGP5(T327C)/pTrx	gp5(T327C)/wt trxA	0.1
pGP5(T327C)/pTrx(C35S)	gp5(T327C)/trx(C35S)	0.07

[a] Plating efficiencies for wild-type T7Δ5 phage were measured as described under "Experimental Procedures." T7Δ5 refers to T7Δ5 phage.

quired 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 displace (42). In general, gp5/trx is poor at strand displacement synthesis as compared with other DNA polymerases, such as ϕ29 DNA polymerase. The ϕ29 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

(43). The precise mechanism as to why gp5/trx is less efficient at strand displacement compared with ϕ29 DNA polymerase is not clear. However, when gp5/trx stalls at sites of secondary structure, presumably there is increased nucleotide turnover (44). Nucleotide turnover involves the gp5/trx catalyzing multiple cycles of nucleotide incorporation and hydrolysis at pause sites. gp5/trx will synthesize through areas of secondary structure if there is a transient duplex opening at the region of secondary structure when the polymerase switches back to polymerization activity (42). Experiments characterizing strand displacement of several DNA polymerases suggest that the 3′-5′ DNA exonuclease activity of a DNA polymerase modulates the ability of the enzyme to strand displace. A gp5/trx complex lacking 3′-5′ DNA exonuclease activity does not have to partition its activities between polymerase and exonuclease activities (41). Thus, gp5/trx lacking exonuclease activity performs strand displacement synthesis more efficiently through duplexed regions than the wild-type enzyme (42). We do not know why gp5-S-S-trx has more difficulty replicating regions of secondary structure than gp5/trx. However, it is possible that the altered polymerase complex strand displaces less efficiently than wild-type gp5/trx complex and thus cannot replicate through these sites. As discussed below, thioredoxin may require more flexibility in its interaction with the polymerase when the complex encounters a hairpin.

Many DNA polymerases that replicate genomic DNA, such as *E. coli* DNA polymerase III and T4 DNA polymerase, utilize a processivity factor that forms a clamp to achieve high processivity (1–5). A crystal structure of gp5/trx has been determined in a ternary complex, with a primer-template in the DNA binding groove and an incoming dideoxynucleotide in the active site positioned for polymerization (17). Earlier mapping of the thioredoxin binding site had placed it on a unique segment of the thumb over the duplex region of the DNA (20, 21). The structure confirmed these predictions but thioredoxin is rotated away from the DNA and does not form a lid over the DNA crevice. We believe that the thumb domain, like the fingers, undergoes conformational changes during a polymerization cycle and we are observing the clamp in an open position. It is tempting to propose that in a processive mode, the flexible binding loop could move down and clamp the thioredoxin and gp5 around the DNA. This model is supported by mutations in thioredoxin that are suppressed by mutations in the exonuclease domain of the polymerase (21), suggesting a possible contact point between the two proteins formed when the polymerase thumb closes thioredoxin over the DNA binding groove to prevent dissociation from the DNA. Alternatively, thioredoxin could help increase the effective electrostatic interaction between the complex and the DNA. This model is supported by mutations affecting basic residues in the thioredoxin binding loop (Lys-300, Lys-302, and Lys-304), which lower the binding affinity of the polymerase for DNA (19).

Regardless of which of the two models for processivity of T7 DNA polymerase is correct one can speculate on the inability of gp5-S-S-trx to polymerize through regions of secondary structure. In the case of wild-type gp5/trx the complex may undergo conformational changes within the thumb subdomain when stalled at secondary structure. Such conformational change would switch the complex into a more processive mode thus enabling nucleotide turnover. This latter reaction occurs with continued binding of the complex to the DNA until conditions allow polymerization through the hairpin by strand displacement synthesis. It is possible that the disulfide linkage between gp5(T327C) and trx(C35S) 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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