Escherichia coli Thioredoxin Stabilizes Complexes of Bacteriophage T7 DNA Polymerase and Primed Templates*

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The DNA polymerase activity induced after bacteriophage T7 infection of Escherichia coli is found in a complex of two proteins, the T7 gene 5 protein and a host protein, thioredoxin. Gene 5 protein is a DNA polymerase and a 3' to 5' exonuclease. Thioredoxin binds tightly to the gene 5 protein and increases the processivity of polymerization some 1000-fold. Gene 5 protein forms a short-lived complex with the primer-template, poly(dA)*oligo(dT), in the absence of Mg\(^2+\) and nucleotides. Thioredoxin increases the half-life of the preformed primer-template-polymerase complex from less than a second to approximately 5 min. The dissociation is accelerated by excess single-stranded DNA in an apparent second order reaction, indicating direct transfer of polymerase between DNA fragments. Thioredoxin also reduces the equilibrium dissociation constant, \(K_d\), of the gene 5 protein-poly(dA)-oligo(dT) complex 20- to 80-fold. The salt dependence of \(K_d\) indicates that thioredoxin stabilizes the primer-template-polymerase complex mainly through additional charge-charge interactions, increasing the estimated number of interactions from 2 to 7. The affinity of gene 5 protein for single-stranded DNA is at least 1000-fold higher than for double-stranded DNA and is little affected by thioredoxin. Under conditions of steady state synthesis the effect of thioredoxin on the polymerization rate is determined by two competing factors, an increase in processivity and a decrease of the dissociation rate of polymerase and replicated template.

*Bacteriophage T7 encodes most of its own DNA replication proteins, including DNA polymerase, the product of gene 5 (Richardson, 1983). A remarkable exception is Escherichia coli thioredoxin, a host protein essential for T7 DNA replication (Mark and Richardson, 1976). In vitro thioredoxin forms a tight complex with gene 5 protein (gp5)\(^+\) in a 1:1 stoichiometry (Modrich and Richardson, 1975a, 1975b). gp5 alone has a high 3' to 5' single-stranded DNA exonuclease activity but very low polymerase and 3' to 5' double-stranded DNA exonuclease activities (Hori et al., 1979a; Adler and Modrich, 1979). Reduced thioredoxin stimulates the latter activities in vitro 50 to 4000-fold, depending on the template used (Hori et al., 1979b; Adler and Modrich, 1979, 1983; Tabor et al., 1987). The single-stranded DNA exonuclease activity of gp5, on the other hand, is not affected by thioredoxin.

Thioredoxin participates in various redox pathways of E. coli by means of its active site disulfide (see Holmgren, 1986). Its only known essential functions are as a subunit of the T7 DNA polymerase (Modrich and Richardson, 1975a) and in the assembly of filamentous phages (Russel and Model, 1986). For both functions the redox capacity of thioredoxin is not required (Huber et al., 1986; Russel and Model, 1986). Both active site cysteines can be replaced without affecting the maximal stimulation of the gp5 polymerase activity. The affinity of these thioredoxin mutants for gp5, however, is severely reduced.

In the accompanying paper (Tabor et al., 1987) we show that the increase in the macroscopic synthesis rate in the presence of thioredoxin is due to an increased processivity of polymerization by the gp5-thioredoxin complex. A primed M13 DNA molecule can be replicated completely without dissociation of the enzyme (Tabor et al., 1986, 1987). gp5 by itself is very nonprocessive and adds only a few nucleotides per association-dissociation cycle. The rate of initiation of replication, on the other hand, is very similar to that observed in the presence of thioredoxin (Tabor et al., 1987).

Functionally, thioredoxin resembles the accessory proteins 44/62 and 45 of the phage T4 DNA polymerase and the auxiliary subunits of the E. coli DNA polymerase III, all of which increase processivity (see Nossal, 1983). The T7 DNA polymerase complex differs from these systems in its simplicity. There is only one accessory protein, thioredoxin, which does not bind to DNA by itself. The gp5-thioredoxin interaction is very stable and salt-insensitive in the absence of DNA (Randahl et al., 1982) and shows an apparent \(K_d\) of less than 5 nM in its presence (Huber et al., 1986). Furthermore, the gp5-thioredoxin complex has no known energy requirement other than for polymerization. We have taken advantage of this simple system to study the effect of thioredoxin on the DNA-binding parameters of T7 gp5. In the current study we have analyzed the stability of preformed enzyme-DNA complexes using poly(dA)-oligo(dT) as a primer-template. Synthesis from single processive cycles has been measured by trapping dissociating polymerase with single-stranded challenger DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA—T7 DNA and T7 [\(^3\)H]DNA (6 cpm/pmol) were prepared as described (Richardson, 1966). Single-stranded M13 mp6 DNA was...
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obtained from H. Nakai (Harvard Medical School) and purified free of contaminating oligonucleotides (Nakai and Richardson, 1986a). Single-stranded M13 [H]DNA (46 cpm/pmol) was prepared as described (Matson and Richardson, 1983). Poly(dA)$_{30}$, oligo(dA)$_{25}$, oligo(dA)$_{18-20}$, oligo(dT)$_{10}$, and the random heteropolymer poly(dA,dC)$_{15}$ were obtained from Pharmacia P-L Biochemicals. The 17-mer poly(A)$_{17}$ was purchased from New England Biolabs. Primer and templates were annealed in a 1:1 molar ratio (for homopolymers) or with a 2-5-fold excess of primer (M13 DNA). Annealing was carried out in 40 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, and 50 mM NaCl by heating to 70 °C for 5 min and slow cooling. Mg$^{2+}$ was subsequently chelated with 20 mM EDTA. Calf thymus DNA (type II) was from Sigma.

**Enzymes and Proteins**—Thioredoxin and T7 gp5 (from E. coli trxA) were both purified from overproducing clones (Tabor et al., 1987). gp5, when assayed in the presence of excess thioredoxin, has Form II polymerase activity (Engler et al., 1986) of approximately 10$^6$ units/mg as previously defined (Modrich and Richardson, 1975b). The concentration of active gp5 molecules was determined in polymerase assays analogous to the one described in Fig. 4. Three dilutions of gp5-thioredoxin were assayed with increasing concentrations of primer-template DNA. The concentration of active gp5 was calculated from the cpm data at half-maximal incorporation, where $E = 2 \times 10^{-6}$, $S$, whereby $E$ and $S$ are the total active enzyme and DNA concentration, respectively. Eliminating $K_D$, we calculate a concentration of 40 nM gp5 in the preincubation mix of the standard polymerase assay. Based on protein determination by Bradford (1976) and Lowry et al. (1951) the values add up to 90 and 110 nM active molecular species of gp5, respectively. All restriction enzymes were purchased from New England Biolabs. Bovine serum albumin was obtained from Miles Laboratories.

**Other Materials**—[H]dTTP (14.8 Ci/mol) was obtained from DuPont-New England Nuclear and diluted with dTTP to 90 cpm/pmol. Unlabeled nucleotides (high performance liquid chromatography grade) were from Pharmacia P-L Biochemicals. DE81 filter discs were purchased from Whatman, nitrocellulose filters (HAWP) from Millipore, and polyethyleneimine-cellulose plates (Polygram) from Brinkmann.

**Methods**

**Nitrocellulose Filter Binding Assay**—Filter binding of double-stranded T7 [H]DNA and single-stranded M13 [H]DNA with either gp5 or gp5-thioredoxin of increasing concentrations followed the protocol of Matson and Richardson (1983), except that buffers did not contain Mg$^{2+}$ or nucleotides. Enzymes and DNA (9.6 μg/ml T7 DNA or 3.2 μg/ml M13 DNA) were preincubated in 50 μl volumes for 10 min at 37 °C. The mixtures were then diluted with the same buffer to give 13.3 μl of 10 μM DNA to trap any free polymerase, and thioredoxin (unless present during preincubation) or 30 μM thioredoxin (if added to the preincubation) or 30 μM thioredoxin (if added with the start mix). All solutions contained in addition 1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5. K$_D$ values were determined by including the DNA in the standard assay except for challenger DNA which was varied from 50 to 400 μg/ml.

**Competition Assay**—Relative binding affinities of gp5 and gp5-thioredoxin for various DNAs were measured by including the DNA to be tested during the preincubation of enzyme and primer-template in the standard polymerase assay. Apparent equilibrium dissociation constants were calculated from the DNA concentration at 50% inhibition of incorporation and the known $K_D$ of the polymerase-poly(dA)oligo(dT) interaction, using the theory of competitive inhibition.

**Salt Dependence of Thioredoxin**—The equilibrium dissociation constants of the interaction of gp5 or gp5-thioredoxin with poly(dA)oligo(dT) were determined at different concentrations of KCl in a variation of the standard assay. The preincubution mix contained in 25 μl: 64 nM poly(dA)oligo(dT), 12-480 nM gp5, 6 μM thioredoxin where desired, and 50-200 mM KCl. The reaction was started with 75 μl of 13.3 mM MgCl$_2$, 0.4 mM [H]dTTP, 20 μg of challenger DNA, 40 μM thioredoxin (unless present during preincubation), and the required amount of KCl. Using its total concentration to 50 μM MgCl$_2$, 0.3 mM [H]dTTP, 200 μg/ml challenger DNA, 16 nM poly(dA)oligo(dT), 3-120 mM gp5, 1.5 or 30 μM thioredoxin, and 50 mM KCl. All solutions contained in addition 0.1 mg/ml BSA, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5. K$_D$ values were determined in Scatchard plots of the equilibrium dissociation constants of 120-480 μg/ml KCl and gp5 concentrations, the contribution of the background to the total incorporation was large. K$_D$ values were therefore also calculated from the initial slopes of the plot "incorporation versus gp5 concentration" using the first derivative with respect to the enzyme concentration [E] and resolving for K$_D$ at [E] = 0: $K_D = [DNA] \times (S_0/S - 1)$, where [DNA] is the total primer-template concentration, $S_0$ is the slope for $K_D = 0$, and S is the experimentally determined slope at zero incorporation. The K$_D$ values obtained from the two methods were consistent within a factor of 1.4 for gp5-thioredoxin and 3 for gp5.

Control experiments confirmed that thioredoxin was saturating at all KCl concentrations and that the thioredoxin-gp5 interaction did therefore not contribute to the observed salt dependence of K$_D$.

**RESULTS**

**Filter-binding Assay of Polymerase-DNA Complexes**—Our initial attempts to determine the effect of thioredoxin on the DNA binding of gp5 made use of the nitrocellulose filter-binding technique (Hinkle and Chamberlin, 1972a). Preliminary experiments showed that with excess gp5 more than 95% of T7 double-stranded [H]DNA and approximately 75% of M13 single-stranded [H]DNA could be retained on the filters. However, a relatively high molar excess of gp5 over DNA was needed in order to trap 50% of the DNA, a 100-fold excess.
over T7 DNA at a DNA concentration of 0.36 nM (expressed in PO4) and a 40-fold excess over M13 DNA at a concentration of 1.4 nM. The addition of thioredoxin to the preincubation of DNA and gp5 surprisingly did not reduce the excess of protein needed but rather lowered the maximal retention of DNA. The results obtained with M13 DNA are shown in Fig. 1. Potential specific binding sites for gp5, consisting of template and 3'-hydroxyl primer terminus, were introduced into T7 DNA by cutting with the restriction enzyme HpaI and by annealing a 17-base oligonucleotide primer to M13 single-stranded DNA. The creation of primer-template structures of random sequence by HpaI cleavage reduced the molar excess of gp5 required for 50% retention of T7 DNA 15-fold (data not shown). However, the annealed primer had no detectable effect on the filter binding of M13 DNA (Fig. 1). The addition of thioredoxin again did not increase the apparent affinity of gp5 for either DNA.

A Preformed Polymerase-DNA Complex on Poly(dA)-oligo(dT)—During preincubation of gp5 and thioredoxin with the primer-template poly(dA)350-oligo(dT)50 in the absence of Mg2+, a stable DNA-protein complex is formed. After the addition of Mg2TTP and excess single-stranded challenger DNA (10 μg of heat-denatured calf thymus DNA) DNA synthesis starts for one processive cycle. Polymerase dissociating from the template after copying it partially or completely is trapped effectively by the challenger DNA and does not reinitiate synthesis on a second poly(dA)-oligo(dT) template (Fig. 2). Synthesis starts without detectable delay after addition of Mg2TTP and a plateau is reached within less than 10 s. This plateau of incorporation is proportional to the number of complexes formed during the preincubation. A standard reaction time of 30 s was chosen. The background of incorporation after 30 s in the presence of challenger DNA is less than 5% (Fig. 2). The processivity of gp5-thioredoxin on poly(dA)-oligo(dT) can be estimated from the final incorporation and the equilibrium dissociation constant of the polymerase-DNA interaction (see below). Assuming that all the gp5 molecules are active (see “Experimental Procedures”), we calculate an incorporation of 170 dTMP residues per gp5. This value is in agreement with the predicted processive synthesis on poly(dA)350-oligo(dT)50 with an average template length of approximately 180 bases. In the absence of challenger DNA, a burst of synthesis by the preformed complexes with an incorporation rate of greater than 100 nucleotides/molecule/second is followed by incorporation at a lower rate (Fig. 2).

In the experiment shown in Fig. 3, gp5 was preincubated with poly(dA)-oligo(dT) in the absence of its accessory protein thioredoxin. After addition of Mg2TTP and challenger DNA, no significant incorporation above background is observed as expected for a distributive polymerase. Alternatively, gp5 may have a very low affinity for the primer-template and thus would not form significant numbers of complexes. Addition of thioredoxin together with Mg2TTP and challenger DNA, however, will rescue preformed gp5-primer-template complexes (Fig. 3A). Rapid initial incorporation of dTMP reaches a plateau within seconds. More than 60% of the added gp5 can be recovered in actively synthesizing complexes, albeit only with a large (4000-fold) molar excess of thioredoxin over gp5 (Fig. 3B). Thioredoxin at a concentration of 800 nM (a 20-fold excess), sufficient to complex more than 99% of gp5 when present during the preincubation, results in less than 5% incorporation when added at the start of the reaction (Fig. 3B). The good recovery of gp5-primer-template complexes at very high concentration of thioredoxin indicates that thioredoxin associates directly with DNA-bound gp5 and that the
binding of thioredoxin has to compete with a high dissociation rate of the preformed gp5-primer-template complex. The final plateau of incorporation is again proportional to the number of preformed complexes as long as synthesis conditions are kept constant.

**Thioredoxin Increases the Affinity of Gene 5 Protein for Poly(dA)·oligo(dT)**—The assay described in the previous section was used to determine the affinity of gp5 for poly(dA)·oligo(dT) in the presence and absence of thioredoxin. In the experiment shown in Fig. 4, gp5 or gp5-thioredoxin was incubated with increasing concentrations of primer-template for 5 min. The reaction was started with MgATP, and challenger DNA was added simultaneously. The incorporation of [3H]dATP after 30 s is shown as a function of the template concentration.

The data obtained with gp5 alone have been analyzed in Scatchard plots (not shown) for the template range of 60-480 nM. Expecting a single binding site per primer-template, the DNA concentration is expressed as 3'-hydroxyl primer termini, an assumption shown to be justified below. From multiple determinations an equilibrium dissociation constant, $K_D$, of 65 ± 11 nM can be calculated for the gp5-primer-template interaction. In the presence of thioredoxin, the $K_D$ becomes too small for classical Michaelis analysis. Instead, the $K_D$ was determined by fitting the data with theoretical titration curves as described by Das and Fujimura (1980). From multiple experiments we calculate a $K_D$ of 3.0 ± 1.3 nM for the interaction of gp5-thioredoxin with poly(dA)·oligo(dT). Thioredoxin thus increases the affinity of gp5 for the primer-template approximately 20-fold in the absence of KCl.

**Affinity of Polymerase for Various DNAs**—The affinity of T7 DNA polymerase for DNA other than poly(dA)·oligo(dT) was determined in competition experiments. The DNA to be tested for binding (competitor DNA) was included during the preincubation of gp5 or gp5-thioredoxin with poly(dA)·oligo(dT). The level of productive enzyme-poly(dA)·oligo(dT) complexes formed in the presence of increasing concentrations of competitor DNA was again monitored in the standard challenger assay. The effect of a variety of DNAs on the binding of gp5 and gp5-thioredoxin to poly(dA)·oligo(dT) is shown in Fig. 5. The binding to the competitor DNA was quantitated by calculating the respective equilibrium dissociation constants, $K_D$ (Table I). The DNA concentration is expressed as the number of potential, nonspecific binding sites (i.e. in bases of single-stranded DNA and in base pairs for double-stranded DNA) (von Hippel et al., 1975) or of potential, specific binding sites (i.e. in primer 3'-hydroxyl ends). The data show that the assumption of a single binding site on poly(dA)·oligo(dT) is justified since neither poly(dA)$_{30}$ nor blunt-end T7 DNA (Table I) is not due to primer-template structures formed on the M13 DNA by contaminating oligonucleotides or poly(dA)$_{30}$.

![Fig. 4. Titration of DNA polymerase with primed template.](image)

**Fig. 4.** Titration of DNA polymerase with primed template. Reaction conditions for the challenged polymerase assay on poly(dA)·oligo(dT) were as described under "Experimental Procedures," except that the primer-template concentration was varied as indicated. Thioredoxin was either present during the preincubation (0) or added at the reaction start together with MgATP and challenger DNA (O). Solid lines represent best fits calculated with a $K_D$ of 70 nM for gp5 and of 3 nM for gp5-thioredoxin. The theoretical titration curve for a $K_D$ of zero is outlined by dots. The background of incorporation into poly(dA)·oligo(dT) in the presence of challenger DNA was determined for each concentration of primer-template and has been subtracted from the values shown.

![Fig. 5. Competition of various DNAs for binding of gp5 in the presence or absence of thioredoxin.](image)

**Fig. 5.** Competition of various DNAs for binding of gp5 in the presence or absence of thioredoxin. Competitor DNA of the indicated concentration was included in the challenged polymerase assay on poly(dA)·oligo(dT) as described under "Experimental Procedures." gp5 (A) or gp5-thioredoxin (B) were preincubated with the poly(dA)$_{30}$·oligo(dT)$_{30}$ primer-template and either poly(dA)$_{30}$ (0), M13 single-stranded DNA (C), T7 double-stranded DNA (A), or Hgal-digested T7 DNA (B). The reaction was started with MgATP, challenger DNA, and thioredoxin (A) or with MgATP and challenger DNA (B) and stopped after 30 s. The competition observed with M13 single-stranded DNA is not due to primer-template structures formed on the M13 DNA by contaminating oligonucleotides or poly(dA)$_{30}$.

![Table I](image)

**Table I**

<table>
<thead>
<tr>
<th>DNA</th>
<th>gp5</th>
<th>gp5-thioredoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (nM)</td>
<td>Non-specific</td>
</tr>
<tr>
<td>Poly(dA)$<em>{30}$·oligo(dT)$</em>{30}$</td>
<td>$25 \times 10^3$</td>
<td>$65 \times 10^3$</td>
</tr>
<tr>
<td>Poly(dA)$_{30}$</td>
<td>$140 \times 10^3$</td>
<td>$370 \times 10^3$</td>
</tr>
<tr>
<td>Oligo(dA)$<em>{30}$·oligo(dT)$</em>{30}$</td>
<td>$150 \times 10^3$</td>
<td>$3000 \times 10^3$</td>
</tr>
<tr>
<td>Poly(dA·dC)$_{15}$</td>
<td>$6 \times 10^3$</td>
<td>$35 \times 10^3$</td>
</tr>
<tr>
<td>M13 single-stranded DNA</td>
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<td>$0.8 \times 10^3$</td>
</tr>
<tr>
<td>T7 double-stranded DNA</td>
<td>$260 \times 10^3$</td>
<td>$11 \times 10^3$</td>
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<tr>
<td>T7 DNA with sticky ends</td>
<td>$4.5 \times 10^3$</td>
<td>$16 \times 10^3$</td>
</tr>
<tr>
<td>T7 DNA with blunt ends</td>
<td>$20 \times 10^3$</td>
<td>$70 \times 10^3$</td>
</tr>
</tbody>
</table>

a DNA concentration expressed in base pairs of double-stranded DNA and in bases of single-stranded DNA, respectively.
b DNA concentration expressed in 3'-termini for double-stranded DNA and in primer 3'-termini for poly(dA)·oligo(dT).
c Not determined.
d T7 double-stranded DNA cleaved with restriction enzymes Hgal or HaeIII.
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4-fold tighter than both poly(dA)-oligo(dT) and blunt-ended DNA (HaeIII restriction fragments). 3) The binding to single-stranded DNA of random sequence (M13 DNA) is 10-300-fold stronger than to the heteropolymer poly(dA,dC) or the homopolymer poly(dA). 4) The low affinity for poly(dA) is due mainly to binding at internal sites and not at the 3' or 5' ends (comparison of poly(dA)\textsubscript{380} and oligo(dA)\textsubscript{40-60}). 5) The binding to double-stranded DNA is negligible. The K\textsubscript{d} values for binding to single-stranded DNA, however, is virtually unaffected. Thioredoxin thus selectively enhances the specific binding of the T7 gp5 to primer termini.

Salt Dependence of the Equilibrium Dissociation Constant—

The salt dependence of the binding of gp5 and gp5-thioredoxin with poly(dA)-oligo(dT) has been determined. The K\textsubscript{d} values given are probably underestimates since they do not account for binding to the ends and nicks of the linear T7 DNA. The binding to nicks has not been quantitated, but introduction of additional nicks by DNase I lowered the apparent K\textsubscript{d} up to 5-fold.

Thioredoxin increases the affinity of gp5 for binding sites with a 3'-hydroxyl primer terminus 20-30-fold. The affinity for single-stranded DNA, however, is virtually unaffected. Thioredoxin thus selectively enhances the specific binding of the T7 gp5 to primer termini.

Dissociation Kinetics of the Preformed Complexes—The half-life of the preformed polymerase-DNA complexes was measured in the following variation of the challenger assay. The significance of this observation is not clear; it may reflect two interconvertible forms of preformed complexes as has been postulated for E. coli DNA.
polymerase I (Bambara et al., 1976) but it is unlikely to be due to a heterogeneity of the gp5-thioredoxin preparation: polymerase isolated from phage-infected cells or from clones overproducing both gp5 and thioredoxin, as well as T7 DNA polymerase Form I (Engler et al., 1983), gave rise to the same effect.

A semilogarithmic plot of the dissociation of the preformed gp5-thioredoxin-poly(dA)-oligo(dT) complex at different concentrations of challenger DNA is presented in Fig. 8. The dissociation rate is strongly dependent on the challenger concentration, with $t_{1/2}$ ranging from 12 to 80 s over an 8-fold concentration range. This result can be explained by direct transfer (von Hippel et al., 1975) of polymerase from the primer-template to the single-stranded challenger DNA. The observed dissociation rate constant can then be expressed as $k_{\text{obsd}} = k_{d} + [c]c' \times k_{o}$, where $c$ is the concentration of challenger DNA, $n$ is the order of the transfer reaction, and $k_{o}$ is the rate constant for direct transfer. Extrapolation of $k_{\text{obsd}} (=\ln(2)/t_{1/2})$ towards zero challenger DNA yields a first order dissociation rate constant, $k_{d}$, of $2.4 \times 10^{-3}$ s$^{-1}$, corresponding to a half-life of the complex of 5 ± 3 min in the absence of DNA-mediated transfer (Fig. 9). This extrapolation assumes that the direct transfer is a first order reaction with respect to challenger DNA. A plot of $\log[c]$ versus $\log(k_{\text{obsd}} - k_{d})$ yields a slope of 1.02, indicating that this is indeed the case (not shown). For the transfer rate constant, $k_{o}$, we then calculate a value of $34 \pm 3$ s$^{-1}$ M$^{-1}$ (DNA concentration expressed in P0$4$).

With gp5 alone the half-life of the binary complex was less than 1 s at all challenger concentrations tested (not shown), so that the contribution of direct transfer to the dissociation rate could not be assessed. However, from the comparison of $t_{1/2}$ at the lowest challenger concentration tested, we conclude that thioredoxin increases the half-life of the gp5-poly(dA)-oligo(dT) complex by at least a factor of 80.

From the equilibrium dissociation constant, $K_{d}$, in the presence of thioredoxin of $3 \times 10^{-3}$ M and the dissociation rate constant, $k_{d}$, of $2.4 \times 10^{-3}$ s$^{-1}$ the association rate constant, $k_{o}$, can be estimated to be approximately $10^{6}$ M$^{-1}$ s$^{-1}$. This is well within the range of diffusion-controlled association rates and does therefore not require facilitated diffusion of the polymerase on single-stranded DNA, at least on poly(dA)-oligo(dT), for primer location (see Berg et al., 1981).

**Cycling of Polymerase**—In the experiments described above polymerase was always restricted to a single processive cycle by means of trapping with challenger DNA. In the following experiments we will analyze some qualitative effects of thioredoxin on the repeated cycling of polymerase under conditions of DNA synthesis. The biphasic kinetics of incorporation into poly(dA)-oligo(dT) in the absence of challenger DNA (Fig. 2) suggests that association or dissociation of enzyme and primer-template may be the rate-limiting step for synthesis by gp5-thioredoxin. The experiment shown in Fig. 10 addresses this question and shows the effect of an additional dissociation step on the steady state incorporation by gp5-thioredoxin into poly(dA)-oligo(dT). gp5-thioredoxin was preincubated with HaeIII-digested T7 DNA at a concentration known from competition experiments to bind greater than 90% of the enzyme. Synthesis was started by the addition of Mg$^{2+}$ and
all four deoxyribonucleotides and, 15 s later, poly(dA)-oligo(dT) (see legend to Fig. 10 for details). Binding to the blunt-ended DNA prior to the addition of poly(dA)-oligo(dT) causes a delay of the steady state incorporation by approximately 10 s compared to the controls. The dissociation of the complex of "replicated" restriction fragment and gp5-thioredoxin apparently is a rate-limiting step, with a half-life of several seconds. In the control reactions, the blunt-ended fragments and primer-template were added together to the polymerase, and the reaction was either started immediately or after a preincubation period. In both cases synthesis starts off at a higher rate before reaching the steady state incorporation, indicating that again dissociation, but not association, is rate limiting. Analogous experiments were carried out with gp5 alone. In the absence of thioredoxin, prior binding of gp5 to the restriction fragments had no effect on the kinetics of incorporation (not shown).

In contrast to the equilibrium experiments described in the previous sections, direct transfer of polymerase between primer-templates will play a role in the kinetics of steady state reactions. We therefore expect a dependence of the synthesis rates on the DNA concentration. The effects of template length and concentration on the macroscopic rate of synthesis by gp5 and gp5-thioredoxin were measured with poly(dA), of variable length, primed with oligo(dT)20. gp5 or gp5-thioredoxin was added to a complete reaction mixture including the primer-template and incorporation was monitored in the linear range. The specific incorporations observed on templates with an average length of approximately 180, 15, and 3 bases, respectively, are summarized in Table II. Three striking results are: 1) the thioredoxin-mediated stimulation of incorporation by gp5 is strongly dependent on template length. In the accompanying paper we show that this length dependence is due to the processivity of the gp5-thioredoxin complex. 2) With very short templates conditions are too short-lived to be retained on the filter. 3) An increase in template concentration stimulates the processive enzyme but not the distributive one, except at the lowest concentration, contrary to what one might expect. These findings, together with the results presented in Fig. 10, can be interpreted as follows. Over most of the template concentration range tested, the association step of gp5 and DNA is not rate limiting; the association rate therefore becomes virtually first order. Since dissociation is not rate limiting either, the limiting step would appear to occur during incorporation or elongation by gp5. For gp5-thioredoxin the rate-limiting step seems to be the dissociation from the primer-template. From the microscopic synthesis rate on poly(dA)180-oligo(dT)20 of more than 100 nucleotides/s and the macroscopic rate of approximately 20 nucleotides/s (Table II), we estimate a half-life of the complex of at least 4 s under synthesis conditions with 200 nM template. Direct transfer would avoid this rate-limiting step and explains the stimulation of synthesis seen with increasing template concentration.

### Discussion

The DNA polymerase activity induced in phage T7-infected *E. coli* cells resides in a complex of the phage gene 5 protein and host thioredoxin (Modrich and Richardson, 1975a, 1975b; Mark and Richardson, 1976). In the accompanying paper (Tabor et al., 1987), we show that the polymerase and exonuclease activities of the complex are inherent properties of gp5. Thioredoxin acts as an accessory protein to increase the processivity of gp5 from a few to several thousand nucleotides. Exactly how the protein-protein interaction increases the processivity is not known. The crystal structure of the large fragment of *E. coli* DNA polymerase I (Ollis et al., 1985a) reveals a large "hand-shaped" binding site for double-stranded DNA. The homology between DNA polymerase I and T7 DNA polymerase (Ollis et al., 1985b) suggests a similar structure for the phage enzyme. Thioredoxin which, by itself, has no affinity for DNA (Huber et al., 1986) may bind near the crevice to induce more specific interactions between gp5 and duplex DNA. In the present study we have exploited the stable binding of thioredoxin and gp5 to measure the effect of the accessory protein on the DNA-binding characteristics of the T7 DNA polymerase.

Nitrocellulose filter-binding assays have been used to measure specific versus nonspecific binding of DNA by a variety of proteins: *E. coli* and phage T7 RNA polymerase (Hinkle and Chamberlin, 1972a, 1972b; Smeekens and Romano, 1986), lac repressor (Winter and von Hippel, 1981), λ repressor (Nelson and Sauer, 1985), and EcoRI restriction endonuclease (Terry et al., 1985), but proved unsuccessful in our hands with the T7 DNA polymerase. We conclude that the contribution by nonspecific binding is too large and/or that specific binding complexes are too short-lived to be retained on the filter.

In this study, therefore, we adopted a polymerase assay with poly(dA)-oligo(dT)20 primer-template by gp5-thioredoxin or gp5 is given in moles of dTMP incorporated per minute of gp5 or gp5-thioredoxin activity. Final reaction mixtures contained: 24 nM gp5 (except in first line: 2.4 nM), thioredoxin (480 nM) and primer-template as indicated, 0.3 mM [H]dTTTP, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 40 mM Tris-HCl, pH 7.5. The reactions were started by the addition of enzyme at 22 °C and stopped with excess EDTA.

<table>
<thead>
<tr>
<th>Template</th>
<th>Concentration</th>
<th>Incorporation rates</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dA)180</td>
<td>6 0.15 53</td>
<td>60 0.7 43</td>
<td>180 0.9 38</td>
</tr>
<tr>
<td>Oligo(dA)40-60</td>
<td>3600 0.7 1.9</td>
<td>7200 0.7 2.8</td>
<td>1800 0.6 136</td>
</tr>
<tr>
<td>Oligo(dA)20-30</td>
<td>1800 0.5 0.5</td>
<td>3600 0.7 1.3</td>
<td>7200 1.5 1.7</td>
</tr>
</tbody>
</table>

* The initial rate of incorporation into poly(dA)40-oligo(dT)20 primer-templates by gp5-thioredoxin or gp5 is given in moles of dTMP incorporated per minute of gp5 or gp5-thioredoxin activity. Final reaction mixtures contained: 24 nM gp5 (except in first line: 2.4 nM), thioredoxin (480 nM) and primer-template as indicated, 0.3 mM [H]dTTTP, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 40 mM Tris-HCl, pH 7.5. The reactions were started by the addition of enzyme at 22 °C and stopped with excess EDTA.

* Ratio of incorporation in the presence versus absence of thioredoxin.
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why thioredoxin mutant proteins with dramatically reduced affinity for gp5 can still support processive synthesis when present in sufficiently high concentration (Huber et al., 1986); dissociating thioredoxin rebinds and stabilizes gp5-DNA complexes before they can decay. The situation is analogous to that of E. coli DNA polymerase III where a large excess of the β subunit can bypass the need for an ATP-stabilized initiation complex (Crute et al., 1983) and also promotes processive synthesis by the polymerase III core enzyme (LaDuca et al., 1986).

Equilibrium measurements of complex formation show that thioredoxin increases the affinity of gp5 for poly(dA)-oligo(dT) approximately 20-fold under our standard conditions. The corresponding equilibrium dissociation constants, \( K_d \), of 65 nM for gp5 and 3 nM for gp5-thioredoxin compare reasonably with the values of 10 nM determined for the processive T5 DNA polymerase (Das and Fujimura, 1980) and 200 nM for E. coli DNA polymerase I (Bryant et al., 1983). At higher salt concentration (75 mM KCl), the stimulation by thioredoxin reaches a maximum of a factor of 80, a value that may still not be sufficient to explain the up to 1000-fold stimulation of synthesis seen with thioredoxin. However, other steps of the incorporation cycle, apart from association-dissociation, are likely to be affected by thioredoxin and to contribute to processivity as well.

The salt dependence of the gp5-poly(dA)-oligo(dT) interaction is very weak compared to other DNA-binding proteins, indicating less than three charge-charge interactions between gp5 and the primer-template. The presence of thioredoxin increases this value to approximately 7 and raises the contribution of the electrostatic interactions to the free energy of complex formation from an estimated 20 to 75%. The salt dependence of the affinity of gp5-thioredoxin for poly(dA)-oligo(dT) and for single-stranded DNA is almost identical. The same result had been obtained with phage T4 DNA polymerase (Newport et al., 1980). The 7–8 charge-charge interactions during binding to single-stranded DNA and to primer-template junctions were taken to suggest that the same electrostatic interactions are involved in both modes of binding. Other DNA-binding proteins displace slightly higher numbers of counterions upon specific and nonspecific binding to double-stranded DNA, respectively: E. coli RNA polymerase holoenzyme, 12 and 9 (Shaner et al., 1982); E. coli lac repressor, 8 and 11 (Winter and von Hippel, 1981), and EcoRI restriction endonuclease, 8 for specific binding (Terry et al., 1985).

As expected from the increase in affinity, thioredoxin also increases the half-life of the gp5-poly(dA)-oligo(dT) complex, from less than 1 s to approximately 5 min. The corresponding dissociation rate constants of greater than 0.7 s\(^{-1}\) in the absence and 2.4 \(\times\) 10\(^{-2}\) s\(^{-1}\) in the presence of thioredoxin can again be compared with those of E. coli DNA polymerase I, 0.25 s\(^{-1}\) (Bryant et al., 1983) and of T5 DNA polymerase, 5.8 \(\times\) 10\(^{-4}\) s\(^{-1}\) (Das and Fujimura, 1980). Both values, measured at a single challenger DNA concentration, do not include possible direct transfer and may therefore be overestimations. A value of 0.3 s\(^{-1}\) has been determined for the actively synthesizing T4 DNA polymerase on single-stranded phage DNA (Mace and Alberts, 1984).

Our results show that single-stranded challenger DNA can accelerate the dissociation of polymerase-primer-template complexes through a mechanism which can best be explained by direct transfer of enzyme between DNA segments (von Hippel et al., 1975). Direct transfer of proteins between DNA domains has also been observed in the case of the E. coli proteins RNA polymerase (Hinkle and Chamberlin, 1972a), cAMP receptor protein, and lac repressor (Fried and Crothers, 1984). The mechanism of direct transfer postulates an intermediate ternary complex, DNA-protein-DNA, requiring two DNA-binding sites. Kinetic data indicate the existence of a second site on DNA polymerase α and on E. coli DNA polymerase I (Fisher and Korn, 1976; Detera and Wilson, 1982). For DNA polymerase I a likely candidate is the 3' to 5' exonuclease site which is located on a different domain, rather distant from the proposed polymerization site (Ollis et al., 1985a). The homology between T7 DNA polymerase and E. coli DNA polymerase I (Ollis et al., 1985b) suggests that the exonuclease site of the T7 enzyme might also serve as a second, discrete DNA-binding site. Interestingly, the reverse transcriptase from HTLV III which lacks an exonuclease activity shows no direct transfer in analogous template-challenge experiments.

During the repeated cycling of the T7 DNA polymerase the slow dissociation of the gp5-thioredoxin-DNA complex apparently is the rate limiting step. The same step has been found to be rate limiting for two other processive DNA polymerases, the T5 polymerase (Das and Fujimura, 1977) and the E. coli DNA polymerase III holoenzyme (Burges and Kornberg, 1983). With gp5 alone neither association nor dissociation are rate limiting under our assay conditions. In this case the rate-limiting step appears to occur during the incorporation cycle, possibly being the slow conformational isomerization of the polymerase-dTTP-primer-template complex as proposed for E. coli DNA polymerase I (Mizrahi et al., 1985). Thioredoxin may accelerate this isomerization step, in addition to its stabilization of the initial polymerase-primer-template complex.

The binding characteristics of T7 DNA polymerase with various DNAs are qualitatively identical to those of E. coli DNA polymerase I (Englund et al., 1969). gp5 binds strongest to primer-template structures, with intermediate strength to internal nonspecific sites on single-stranded DNA and very little, if at all, to double-stranded DNA. The accessory protein thioredoxin specifically stimulates the affinity of gp5 for primer-template junctions, resulting in two major, counteracting effects on the macroscopic synthesis rates: 1) an increase in processivity, which dominates on long templates; and 2) a decreased dissociation rate of the polymerase-replicated template complex which, on short templates and at low template concentration, can cancel the gains from processivity.

gp5 and thioredoxin are but two of the seven proteins required for the initiation and bidirectional replication of the T7 genome in vitro (Romano et al., 1983; Englund and Richardson, 1983; Fuller and Richardson, 1985). The number of proteins involved in vivo probably is even greater. In vitro, a replication fork can be driven by a single additional protein, gene 4 protein (gp4), which is both a DNA helicase and primase. The interactions of gp5-thioredoxin, gp4, and DNA have been investigated in detail by Nakai and Richardson (1986a, 1986b).

How do the DNA-binding properties of gp5-thioredoxin fit into the model of coupled leading and lagging strand synthesis in a replication fork (Sinha et al., 1980)? The model postulates that the two replication protein complexes, consisting of at least the DNA polymerase and helicase, migrate coordinate along the two strands of the fork. Our results show that neither gp5 nor gp5-thioredoxin exist as dimers on prized single-stranded DNA, since virtually 100% of gp5 can be found in synthetically competent complexes. Dimers may be stabilized in a replication fork, however, with each monomer

\(^8\) H. E. Huber and C. C. Richardson, unpublished material.
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binding to one DNA strand. Alternatively, gp4 or another component of the replication machinery may mediate dimer formation. gp4 seems to be a promising candidate because protein-protein interaction between gp5-thioredoxin and gp4 has been observed in vitro (Nakai and Richardson, 1986a).

The thioredoxin-mediated affinity of gp5 for primer-templates results in the desirable processivity of synthesis on the leading strand. On the other hand, the repeated cycling of the polymerase on the lagging strand would seem to be severely handicapped by thioredoxin. In the case of phase T4 DNA polymerase and E. coli DNA polymerase III, a release of the accessory proteins, possibly concomitant with ATP-hydrolysis, is plausible. For the T7 DNA polymerase a release of the tightly bound thioredoxin seems highly improbable. However, the DNA-mediated dissociation of the gp5-thioredoxin-primer-template complex might provide a very efficient mechanism for the facilitated release and direct transfer of polymerase from a completed Okazaki fragment to the next primer. Recent experiments in our laboratory with preformed replication forks show that a preformed, but not an actively synthesizing, complex of gp6-thioredoxin and gp4 can be challenged with single-stranded DNA. This result suggests, as required of such a mechanism, that only a pausing polymerase would be susceptible to direct transfer.

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REFERENCES


*H. Nakai and C. C. Richardson, manuscript in preparation.*