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Amino Acid Residues Critical for the Interaction between Bacteriophage T7 DNA Polymerase and Escherichia coli Thioredoxin

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Upon infection of Escherichia coli, bacteriophage T7 annexes a host protein, thioredoxin, to serve as a processivity factor for its DNA polymerase. T7 gene 5 protein. In a previous communication (Himawan, J., and Richardson, C. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9774–9778), we reported that an E. coli strain encoding a Gly-74 to Asp-74 (G74D) thioredoxin mutant could not support wild-type T7 growth and that in vivo, six mutations in T7 gene 5 could individually suppress this G74D thioredoxin defect. In the present study, we report the purification and biochemical characterization of the G74D thioredoxin mutant and two suppressor gene 5 proteins, a Glu-319 to Lys-319 (E319K) mutant of gene 5 protein and an Ala-45 to Thr-45 (A45T) mutant. The suppressor E319K mutation, positioned within the DNA polymerization domain of gene 5 protein, appears to suppress the parental thioredoxin mutation by compensating for the binding defect that was caused by the G74D alteration. We suggest that the Glu-339 to Lys-339 (E339K) mutation in the T7 gene 5 protein and the Gly-74 residue of E. coli thioredoxin define a contact point or site of interaction between the two proteins. In contrast, the A45T mutation in gene 5 protein, located within the 3’ to 5’ exonuclease domain, does not suppress the G74D thioredoxin mutation by simple restoration of binding affinity. Based upon our understanding of the mechanisms of suppression, we propose a model for the T7 gene 5 protein-E. coli thioredoxin interaction.

The concept of using genetic or suppressor analysis to investigate protein-protein interaction can be described as follows. If two proteins form a complex, then there must exist a contact point, or more likely, several contact points between them. These contact points would be defined by certain amino acid residues of one protein that must be physically adjacent to certain amino acid residues of the other protein. If a contact point amino acid from one protein is structurally altered significantly by mutation, then complex formation with the second protein would be destroyed. Theoretically, a productive complex could be formed once again by an alteration in the second protein that structurally compensates for the original mutation. Therefore, by mutating one protein of a complex and selecting for extragenic suppressor mutations in the second protein, one should be able to identify the contact points between the two proteins.

We (1) have used extragenic suppressor analysis to investigate the interaction between two proteins that are involved in DNA replication in E. coli infected with bacteriophage T7. Similar to our studies, other workers have also used suppressor analysis to study protein-protein interactions in the E. coli DNA replication system (2) and also in the DNA replication system of the yeast Saccharomyces cerevisiae (3). Specifically, we have been investigating by suppressor analysis the interaction between T7 gene 5 protein and E. coli thioredoxin. T7 gene 5 protein, the DNA polymerase of phage T7 (4, 5), has two enzymatic activities: a nonprocessive 5’ to 3’ DNA polymerase activity (6–8) and a 3’ to 5’ exonuclease activity (7, 9). During infection of E. coli, T7 annexes the host protein thioredoxin, a general protein disulfide oxidoreductase (10), as a processivity factor for polymerization of nucleotides (8, 11–14). Thioredoxin also greatly stimulates the 3’ to 5’ exonuclease activity of T7 gene 5 protein on double-stranded DNA (7, 9).

We have focused our studies on the interactions between T7 gene 5 protein and thioredoxin in order to understand how thioredoxin confers processivity upon gene 5 protein. Unlike other more complex DNA replication systems, such as those of bacteriophage T4, E. coli, and eukaryotes (15–18), the relative simplicity and experimental tractability of the phage T7 DNA replication system affords the attractive possibility of understanding processivity comprehensively at a molecular level. In this work, we have complemented our previous genetic analysis of the interaction between T7 gene 5 protein and E. coli thioredoxin (1) with a biochemical analysis of the same interaction. We began our previous investigation by using E. coli thioredoxin mutants that were unable to support wild-type (WT) T7 growth to select for suppressor strains of phage T7 that contained a compensating mutation in gene 5. We found that an E. coli strain containing a glycine 74 to aspartate 74 substitution (G74D) in thioredoxin could not support the growth of WT T7 phage. Furthermore, we found that six different mutations in T7 gene 5 could individually suppress the G74D alteration in thioredoxin. Three of the six suppressor mutations (E319K, E319V, Y409C) were positioned within the putative DNA polymerization domain of T7 gene 5 protein, and the other three suppressor mutations (A45T, V3I, V32A) were positioned...
within the 3' to 5' exonuclease domain. In this paper, we have attempted to explain, at a biochemical level, the mechanism of suppression for two representative suppressor mutations (E319K and A45T).

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Bacteriophage Strains, and Plasmids—E. coli JH20 (ΔtrxA307, pC880), E. coli HMS321 (trxA5, pC880), E. coli SB2111 (a strain harboring the plasmid pBR325trxA11), E. coli MV110 (EB300 supE, supF, supF1, supF2, supF3, supF4, supF5, supF6) and WT bacteriophage T7, phase T7trxA (a T7 phage that has a WT thioredoxin gene inserted in its genome), phase T7-5-E319K, phase T7-5-A45T, bacteriophage PI1, and plasmid pBR325trxA11 (a plasmid encoding a Gly-74 to Asp-thioredoxin mutant) were constructed previously (1). E. coli A307,ompT, a derivative of E. coli A307 (19) that contains an additional mutation in the ompT gene, was used. Plasmid pA307 (20), both derivatives of pG35–5, were constructed as described below.

E. coli AN1 (ΔtrxA307, pC880) was constructed by using phage PI1 to transduce the ΔtrxA307 allele from JH20 into HMS321. The presence of the thioredoxin deletion (ΔtrxA307) within AN1 was verified by several methods. First, AN1 was unable to support the growth of WT T7 but was able to support the growth of T7trxA, indicating a defect in the thioredoxin gene. Second, amplification of chromosomal DNA by PCR of the chromosomal DNA surrounding the thioredoxin gene showed the presence of a deletion of the appropriate size. Third, restriction enzyme analysis of the PCR-generated DNA fragment confirmed that the deletion was positioned correctly.

E. coli AN1(pBR325trxA11), the strain used to purify the Gly-74 to Asp-thioredoxin (G74DTrxA), was constructed by purifying the plasmid pBR325trxA11 from E. coli SB2111 and by transforming pBR325trxA11 into E. coli AN1. Strain SB2111 was not used for the production of the G74DTrxA, because the pcnB mutation of SB2111 reduces the intracellular copy number of plasmid pBR325trxA11 (1). Consequently, SB2111 produces less G74DTrxA than AN1(pBR325trxA11).

**Materials**—AmpliTaq DNA Polymerase, purchased from Perkin-Elmer, was used for in vitro DNA amplification by PCR (21). Unless specified otherwise, all oligonucleotides used in this work were provided by Dr. Alex Nussbaum (Harvard Medical School). Nonradioactive nucleic acids were purchased from a U.S. Biological Corp. and that contains the T7 RNA polymerase gene under the transcriptional control of the lac promoter (8), and plasmid pG35–5, a derivative of plasmid pG35–5, was purchased from Pharmacia Biotech Inc. [35S]dATP was purchased from Amersham Life Science. The restriction enzymes PshAI and HhaI were purchased from Takara Biochemical Inc. All restriction enzymes were used according to the manufacturers’ instructions and were equilibrated with buffer A. Bound G74D thiorexin was eluted by a 250-mM continuous gradient of NaCl (25 mM to 500 mM) in buffer B. The fractions containing thioredoxin were pooled (70 ml), concentrated by ammonium sulfate precipitation (33 g/70 ml), and dissolved in 1 ml of 10 mM Tris–Cl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol (buffer A) and was applied onto a DEAE-cellulose column (4.9 cm × 8 cm) that was equilibrated in buffer B. Thioredoxin bound to the Mono Q matrix and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Using the fast protein liquid chromatography (FPLC) system from Pharmacia, fraction V (5 ml in buffer B) was loaded onto a Mono Q 5/5 column (1-ml bed volume) that was equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, concentrating ammonium sulfate precipitation (33 g/70 ml), and dissolved in 1 ml of 10 mM Tris–Cl, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol (buffer B) to generate fraction IV. Fraction IV (1 ml) was subjected to gel filtration through a Sephadex G-50 column (1.2 cm × 50 cm) that was equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. The amount of protein in a given solution was determined by the method of Bradford (31), using the Bio-Rad protein assay kit and using bovine plasma γ globulin (Bio-Rad) as a standard protein. An ice-cold solution containing 50 mM Tris–Cl, pH 7.5, 5 mM dithiothreitol, and 0.5 μg/ml bovine serum albumin was used to dilute enzymes immediately prior to the desired reactions. Polyacrylamide gel electrophoresis followed by Coomassie staining was performed by standard techniques (28), and polyacrylamide gel electrophoresis followed by silver-staining was performed using the PhastSystem from Pharmacia.

Expression and Purification of the Gly-74 to Asp-thioredoxin (G74D TrxA)—E. coli strain AN1, harboring the plasmid pBR325trxA11, was used to produce the G74D thioredoxin. By slightly modifying previous protocols that were used to purify WT thioredoxin (11, 19, 32, 33), we developed a six-step, three-column procedure to purify the G74D mutant thioredoxin. Throughout this purification procedure, all solutions containing the G74D thioredoxin were kept at 0–4°C. Two liters of E. coli AN1(pBR325trxA11) were grown in LB media (2%) Tryptone, 1% yeast extract, and 0.5% NaCl) with vigorous shaking. Following overnight growth, the cell culture was centrifuged to collect the cells, and the cells (wet weight of 10 g) were resuspended in ice-cold buffer containing 50 mM Tris–Cl, pH 8.2, 20 mM EDTA, 10% sucrose. The solution of resuspended cells was frozen by treatment with liquid nitrogen and the frozen cells were thawed by an overnight incubation at 0°C. This freeze-thaw procedure selectively releases thioredoxin from the cells (34). The cell debris was removed by centrifugation to produce fraction I (freeze-thaw supernatant). Residual amounts of DNA were removed from fraction I (35 ml) by the addition of streptomycin sulfate (4% final concentration), allowing selective precipitation of the DNA by centrifugation to generate fraction II (streptomycin sulfate supernatant). Ammonium sulfate was added to fraction II (3.5 g/70 ml) to precipitate proteins, generating fraction III (ammonium sulfate supernatant). Fraction III (35 ml) was dialyzed against 2 liters of 10 mM Tris–Cl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol (buffer A) and was applied onto a DEAE-cellulose column (4.9 cm × 8 cm) that was equilibrated in buffer B. Thioredoxin bound to the Mono Q matrix and was eluted by a continuous gradient of NaCl (10–300 mM) in buffer B. Under these conditions, thioredoxin (fraction VI) was eluted by a 250-mM continuous gradient of NaCl (25 mM to 500 mM) in buffer B. The fractions containing thioredoxin were pooled (70 ml), concentrated by ammonium sulfate precipitation (33 g/70 ml), and dissolved in 1 ml of 10 mM Tris–Cl, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol (buffer B) to generate fraction IV. Fraction IV (1 ml) was subjected to gel filtration through a Sephadex G-50 column (1.2 cm × 50 cm) that was equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V. Under these conditions, thioredoxin (fraction VI) eluted at 85 mM NaCl, equilibrated in buffer B and fractionsthat contained thioredoxin were pooled (5 ml) to generate fraction V.
The plasmid pGP5–5 was incubated with T4 DNA ligase, generating the plasmid pGP5–5-E319K. The expression and purification of the mutant A45Tg5P were performed by the same procedure described for the E319Kg5P. A 2-liter culture of E. coli A307, ompT(pGP5–5–A45T) cells was grown, and 5 g of cells (wet weight) were harvested. The purification of the A45Tg5P from this 5 g of cells is summarized in Table I. A45Tg5P was purified approximately 700-fold with a total yield of 12%. Fraction IV was estimated to be 90% pure, as judged by denaturing polyacrylamide gel electrophoresis.

DNA Polymerase Assays—DNA polymerase activity was measured by a modification of previous procedures (4–6). DNA polymerase assays were performed using either heat-denatured (4) calf thymus DNA (Sigma) or simply primed M13mp18 DNA as a template. Primed M13mp18 DNA was made by annealing (8) an oligodeoxynucleotide (5'-CTGGTTTCAAAGATCCGACAC-3' from U.S. Biochemical Corp.) to M13mp18 DNA (U.S. Biochemical Corp.).

DNA polymerase activity measured the amount of [\textsuperscript{14}C]dTMP incorporated into DNA during incubation at 37°C in a buffer containing 2 mM MgCl\(_2\), 5 mM dithiothreitol, 50 mM KCl, 1500 pmol (in nucleotide equivalent) of each dNTP (dATP, dCTP, dGTP, and [\textsuperscript{14}C]dTTP to a specific activity of approximately 5 cpmp/mol), 50 mM NaCl, 10 mM MgCl\(_2\), 40 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, and either 100 \mu \text{m} of heat-denatured calf thymus DNA or 20 \mu \text{m} of primed M13mp18 DNA as a template. Reactions were terminated by adding EDTA to a final concentration of 50 mM, and the reaction mixture was then placed on DE81 ion exchange paper. Unincorporated nucleotides were removed by washing the paper in a solution of 500 mM Na\textsubscript{2}HPO\(_4\), pH 7.0 (28), at 25°C for 1 h, with a change into fresh washing solution every 20 min. The washed paper was dried under a heat lamp, and the amount of radioactivity bound to the paper was measured by liquid scintillation counting. Under these conditions, a unit of DNA polymerase activity was defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of deoxynucleotides into DNA at 37°C in 30 min.

The amount of protein and the units of DNA polymerase activity were determined as described under “Experimental Procedures.”

### Table I

| Fraction and step | Protein | Units | Specific activity | Recovery |
|------------------|---------|-------|------------------|----------|
|                   | mg      | \times 10^3 | units/mg | %       |
| **I. Extract**   | 1800    | 900   | 500            | 100      |
| **II. Ammonium sulfate** | 1600    | 460   | 290            | 51       |
| **III. DEAE-cellulose** | 350     | 360   | 1030           | 40       |
| **IV. Phosphocellulose** | 3       | 50    | 16,700        | 6        |
| **V. DEAE-Sepharose A-50** | 1.5     | 40    | 26,700        | 4        |

**Purification of the A45T gene 5 protein**

| Fraction and step | Protein | Units | Specific activity | Recovery |
|------------------|---------|-------|------------------|----------|
|                   | mg      | \times 10^3 | units/mg | %       |
| **I. Extract**   | 600     | 49    | 81              | 100      |
| **II. Ammonium sulfate** | 600     | 49    | 81              | 100      |
| **III. DEAE-cellulose** | 15      | 20    | 1347           | 42       |
| **IV. Phosphocellulose** | 0.1     | 5.8   | 58,000         | 12       |

**Purification of the E319K gene 5 protein**

| Fraction and step | Protein | Units | Specific activity | Recovery |
|------------------|---------|-------|------------------|----------|
|                   | mg      | \times 10^3 | units/mg | %       |
| **I. Extract**   | 1800    | 900   | 500            | 100      |
| **II. Ammonium sulfate** | 1600    | 460   | 290            | 51       |
| **III. DEAE-cellulose** | 350     | 360   | 1030           | 40       |
| **IV. Phosphocellulose** | 3       | 50    | 16,700        | 6        |
| **V. DEAE-Sepharose A-50** | 1.5     | 40    | 26,700        | 4        |

The plasmid pGP5–5 was incubated with T4 DNA ligase, generating the plasmid pGP5–5-E319K. The expression and purification of the mutant A45Tg5P were performed by the same procedure described for the E319Kg5P. A 2-liter culture of E. coli A307, ompT(pGP5–5–A45T) cells was grown, and 5 g of cells (wet weight) were harvested. The purification of the A45Tg5P from this 5 g of cells is summarized in Table I. A45Tg5P was purified approximately 700-fold with a total yield of 12%. Fraction IV was estimated to be 90% pure, as judged by denaturing polyacrylamide gel electrophoresis.

DNA Polymerase Assays—DNA polymerase activity was measured by a modification of previous procedures (4–6). DNA polymerase assays were performed using either heat-denatured (4) calf thymus DNA (Sigma) or simply primed M13mp18 DNA as a template. Primed M13mp18 DNA was made by annealing (8) an oligodeoxynucleotide (5'-CTGGTTTCAAGATCCGACAC-3' from U.S. Biochemical Corp.) to M13mp18 DNA (U.S. Biochemical Corp.).

DNA polymerase activity measured the amount of [\textsuperscript{14}C]dTMP incorporated into DNA during incubation at 37°C in a buffer containing 2 mM Mg\(_2\), 5 mM dithiothreitol, 300 \mu \text{m} of each dNTP (dATP, dCTP, dGTP, and [\textsuperscript{14}C]dTTP to a specific activity of approximately 5 cpmp/mol), 50 mM NaCl, 10 mM MgCl\(_2\), 40 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, and either 100 \mu \text{m} of heat-denatured calf thymus DNA or 20 \mu \text{m} of primed M13mp18 DNA as a template. Reactions were terminated by adding EDTA to a final concentration of 50 mM, and the reaction mixture was then placed on DE81 ion exchange paper. Unincorporated nucleotides were removed by washing the paper in a solution of 500 mM Na\textsubscript{2}HPO\(_4\), pH 7.0 (28), at 25°C for 1 h, with a change into fresh washing solution every 20 min. The washed paper was dried under a heat lamp, and the amount of radioactivity bound to the paper was measured by liquid scintillation counting. Under these conditions, a unit of DNA polymerase activity was defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of deoxynucleotides into DNA at 37°C in 30 min.

Exonuclease Assays—3′ to 5′ exonuclease activity was measured by modifying previously described procedures (36, 40). Reaction conditions for the exonuclease assay contained 40 mM Tris-Cl, pH 7.5, 10 mM MgCl\(_2\), 5 mM dithiothreitol, 50 mM KC\(_2\), 1500 pmol (in nucleotide equiv-
alents) of double-stranded (6 nm) or single-stranded (12 nm) $[^3]H$DNA, 3 nm gene 5 protein, and, when present, 1 μM thioredoxin. The reaction mixture was incubated at 37 °C in a total volume of 100 μl, and the reactions were terminated by adding EDTA to a final concentration of 50 mM. DNA was then precipitated by adding 30 μl of an ice-cold solution of bovine serum albumin (10 mg/ml) and 30 μl of an ice-cold solution of trichloroacetic acid (100% w/v). After a 20-min incubation on ice, the precipitated DNA was removed by centrifugation at 12,000 × g for 20 min at 4 °C. The amount of radioactivity in 125 μl of the resulting supernatant was measured by liquid scintillation counting using 6 ml of Ultima Gold (Packard Instrument Co.) as a fluor.

Double-stranded ($[^3]H$DNA was synthesized by PCR. The reaction mixture (100 μl) contained 20 μl Tris-Cl, pH 8.3 at 20 °C, 1.5 mM MgCl$_2$, 25 mM KCl, 0.05% Tween 20, 100 μg/ml bovine serum albumin, 30 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 5 units of AmpliTaq DNA polymerase, 20 pmol of each primer, approximately 10$^6$ template molecules, and 10 μl of $[^3]H$dTTP (43 Ci/mmol, 1 μCi/ml). The amplified product (a 1200-base pair, linear DNA fragment) was purified by the Geneclean kit. The specific activity of the ($[^3]H$DNA was typically between 10 and 50 cpm/pmol in nucleotide equivalents. Single-stranded ($[^3]H$DNA was prepared by heating the labeled double-stranded DNA at 100 °C for 5 min, followed by immediate chilling on ice.

Measuring Binding Between T7 Gene 5 Protein and E. coli Thioredoxin—Binding affinity between gene 5 protein and thioredoxin was measured as described previously (36). Gene 5 protein and thioredoxin, purified separately, were reconstituted in solution, and productive binding was monitored by measuring DNA polymerase activity. The computer program KaleidaGraph (developed by Abelbeck Software) was used to construct a Scatchard plot (37) on the ordinate to calculate a correlation coefficient ($r$) that measures how well the given data fit the generated line. A correlation coefficient of 1 ($r = 1$) would mean that the data produced a line perfectly.

Processivity Assays—Processivity of polymerization was measured by a modification of a previously described procedure (8). An oligodeoxyoxynucleotide (5'-GTTTCCCCAGTCACGAC-3') complementary to M13mp18 DNA was labeled with $[^3]P$ at its 5'-end using T4 polynucleotide kinase. The $[^3]P$-5'-end labeled oligonucleotide was annealed to M13mp18 DNA (8), and the 5'-labeled primed M13mp18 DNA was purified by using the Geneclean kit. DNA synthesis was carried out in a reaction that contained 300 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 40 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$, 50 mM NaCl, 5 mM dithiothreitol, 20 mM 5'-labeled primed M13mp18 DNA, 2 nm gene 5 protein, and 2 μM thioredoxin. The reaction mix was preincubated for a minute at 37 °C without proteins, and the reaction was started by the addition of the proteins. At various times after the start, the reaction was terminated by adding EDTA to a final concentration of 50 mM, and the products of the reaction were analyzed by electrophoresis through a 0.8% agarose gel containing ethidium bromide (0.5 μg/ml). The gel was then dried, and the location of the radioactivity was determined by autoradiography.

### RESULTS

We (1) developed a genetically based method to probe the interactions between the bacteriophage T7 DNA polymerase, product of gene 5, and its processivity factor, E. coli thioredoxin. We used an E. coli strain harboring a Gly-74 to Asp-74 (G74D) thioredoxin mutation that was unable to support WT T7 growth. We constructed a template as described under “Experimental Procedures.” Specific activity of DNA polymerase was determined as pmol of deoxynucleotide incorporated into DNA per ng of protein per min. The activity values in this table are average values from three experiments. S.D. of the numerical sample is shown in parentheses.

| Gene 5 protein-thioredoxin complex | Specific activity (S.D.) |
|-----------------------------------|------------------------|
| WT-WT                             | 20 (1)                 |
| WT-G74D                           | 3 (1)                  |
| E319K-WT                          | 74 (11)                |
| E319K-G74D                        | 51 (2)                 |
| A45T-WT                           | 56 (2)                 |
| A45T-G74D                         | 8 (3)                  |

### TABLE II

DNA polymerase activity of the various gene 5 protein-thioredoxin complexes.

### RESULTS

We (1) developed a genetically based method to probe the interactions between the bacteriophage T7 DNA polymerase, product of gene 5, and its processivity factor, E. coli thioredoxin. We used an E. coli strain harboring a Gly-74 to Asp-74 (G74D) thioredoxin mutation that was unable to support WT T7 growth to select for suppressor T7 strains that contained compensatory mutations in its gene 5. Six different mutations in T7 gene 5 are individually necessary and sufficient to compensate for the G74D thioredoxin defect. Three suppressor mutations (E319K, E319V, and Y409C) reside within the polymerization domain of T7 DNA polymerase, and the other three suppressor mutations (V3I, V32A, and A45T) reside within the 3'- to 5' exonuclease domain. In order to understand the mechanism of suppression, we have biochemically characterized the G74D thioredoxin and two of the mutant gene 5 proteins, E319K and A45T, representatives of suppressor mutations in the polymerase and exonuclease domains, respectively. G74D thioredoxin (G74DTrxA), E319K gene 5 protein (E319K-G5p), and A45T gene 5 protein (A45T-G5p) were purified to apparent homogeneity from E. coli cells expressing the mutant genes as described under “Experimental Procedures.” DNA Polymerase Activity—We began our biochemical characterization by reconstituting the three gene 5 proteins (WT, E319K, and A45T) with the two thioredoxins (WT and G74D) in all six possible combinations and by measuring the DNA polymerase activity of each gene 5 protein-thioredoxin complex. These experiments were performed to compare quantitatively the activities of the various mutant complexes and also to evaluate qualitatively the interaction between a particular gene 5 protein and a corresponding thioredoxin. The results are summarized in Table II.

Both mutant gene 5 proteins, when complexed with the mutant G74D thioredoxin, were more active (3-fold for the A45T-G5p and 17-fold for the E319K-G5p) than WT gene 5 protein complexed to the same mutant thioredoxin. This result was expected since the A45T and E319K mutations in gene 5 were isolated as suppressors of the G74D thioredoxin mutation (1). From the data in Table II, it is evident that the A45T and E319K mutations are not allele-specific for DNA polymerase activity in vitro, because both mutant gene 5 proteins are able to form an active DNA polymerase when complexed to either the mutant thioredoxin or the WT thioredoxin. In fact, both mutant gene 5 proteins had more polymerase activity when complexed to the WT thioredoxin than when complexed to the mutant thioredoxin. This in vitro result is consistent with the in vivo results since E. coli strains encoding WT thioredoxin are able to support the growth of both suppressor phage strains, T7-3-A45T and T7-3-E319K.

Binding Affinity—To quantitatively assess the interaction between a given gene 5 protein and a particular thioredoxin,
The purified gene 5 proteins (WT, E319K, and A45T) were mixed in a DNA polymerase activity assay. The amount of DNA synthesis was measured as described under "Experimental Procedures," and the data were generated from a single experiment. Therefore, the32-fold increase in the equilibrium dissociation constant (K_{obs}) for the G74D thioredoxin defect. Evidently, the A45Tg5P mutation is suppressing the G74D thioredoxin mutation by a more complicated mechanism. Since the A45Tg5P mutation is located within the putative 3' to 5' exonuclease domain of T7 gene 5 protein (1, 40), the 3' to 5' exonuclease activity of all the proteins was evaluated (see below).

It should be noted that Huber et al. (36), using the same procedure, also measured the binding affinity between WT T7 gene 5 protein and WT thioredoxin (36). As shown in Table III, we calculated the K_{obs} of the interaction between WT gene 5 protein and WT thioredoxin to be 400 nM at 37°C. In contrast, Huber et al. (36) reported values of 5 nM at 30°C and 25 nM at 42°C for the same interaction. We attribute this approximately 20-fold difference in binding affinity primarily to differences in template and protein preparation, both of which can potentially affect the results quite significantly. To circumvent these problems, all measurements reported in this work were made using identical template and buffer preparations to ensure internal relative consistency throughout the course of this study.

Exonuclease Assays—The 3' to 5' exonuclease activity of all six T7 gene 5 protein-thioredoxin complexes was determined, and the results are summarized in Table IV. As expected, the A45Tg5P, harboring a mutation in the putative exonuclease domain of the protein, had reduced exonuclease activity (11%) as compared with the WT activity with single-stranded DNA as a substrate. Even when complexed to thioredoxin, the A45Tg5P had reduced exonuclease activity as compared with the WTg5P.

With single-stranded DNA as a substrate, the E319Kg5P also had reduced exonuclease activity (28%) as compared with the WT activity. However, when complexed with the WT thioredoxin, the E319Kg5P had only slightly less activity (84%) than the WTg5P-WTrxA complex, and when bound to the mutant G74D thioredoxin, the E319Kg5P had approximately 5-fold more activity than the WTg5P bound to the same mutant thioredoxin. As with the results of the binding assay, this last result suggests that the E319K mutation restored the interaction with the G74D thioredoxin.

The 3' to 5' exonuclease activities of the three gene 5 proteins (uncomplexed to thioredoxin) with double-stranded DNA as substrate are not shown, because gene 5 protein alone has negligible exonuclease activity on double-stranded DNA substrates (7, 9). Similarly, the exonuclease activities of the various gene 5 protein-thioredoxin complexes on a single-stranded DNA substrate are not shown, since thioredoxin stimulates...
Processive than the WTg5P-G74DTrxA complex, then there in this assay. If the A45Tg5P-G74DTrxA complex was more does
lanes 10–12

expected, the E319K mutation in gene 5 does increase the ability protein to interact with the WT thioredoxin; however, as ex-
son of

the ability of thioredoxin to confer processivity upon gene 5

only slightly the exonuclease activity of gene 5 protein on single-stranded substrates (7, 9).

Processivity of Polymerization—The ability of thioredoxin to

stimulate the DNA polymerase activity of T7 gene 5 protein (6, 7) is due primarily to an increase in the processivity of nucleotidyl polymerization by gene 5 protein (8, 14). Therefore, the processivity of each gene 5 protein-thioredoxin complex was examined to determine if the gene 5 and thioredoxin mutations (E319K and A45T in gene 5 and G74D in thioredoxin) affected the ability of thioredoxin to confer processivity upon gene 5 protein. Processivity of nucleotide polymerization was examined as described under “Experimental Procedures.” A $^{32}$P-5'-end-labeled oligodeoxynucleotide was annealed to single-stranded M13mp18 DNA, and DNA synthesis was carried out using a 10:1 molar ratio of primer-template to enzyme (gene 5 protein-thioredoxin). For each gene 5 protein-thioredoxin complex, the polymerization reaction was terminated at 1, 5, and 15 min, and the reaction products were examined by electrophoretic separation through an agarose gel. The results are shown in Fig. 2.

A comparison of lanes 1–3 and lanes 10–12 shows that the G74D thioredoxin mutation decreases the ability of thioredoxin to confer processivity upon the WT gene 5 protein. In fact, in this assay, processive nucleotide polymerization by the WTg5P-G74DTrxA complex is undetectable (lanes 10–12). A comparison of lanes 1–3 and lanes 4–6 shows that the E319K mutation in gene 5 does not significantly alter the ability of gene 5 protein to interact with the WT thioredoxin; however, as expected, the E319K mutation in gene 5 does increase the ability to interact with the G74D mutant thioredoxin (compare lanes 10–12 with lanes 13–15).

Since the A45T mutation in gene 5 reduced the 3' to 5' exonuclease activity (Table IV) and did not restore binding to the G74D thioredoxin (Table I), we considered the possibility that it was compensating for the thioredoxin defect by altering the processivity of nucleotide polymerization. A comparison of lanes 10–12 and lanes 16–18 of Fig. 2 shows that the A45Tg5P does not detectably increase the processivity of polymerization in this assay. If the A45Tg5P-G74DTrxA complex was more processive than the WTg5P-G74DTrxA complex, then there should be more DNA synthesis in lanes 16–18 as compared with lanes 10–12. However, there is no obvious difference between lanes 10–12 and lanes 16–18, suggesting that the A45T mutation in gene 5 protein does not suppress the G74D thioredoxin defect by increasing the processivity of nucleotide polymerization. This experiment was repeated several times under slightly different conditions, and in each case, no difference in processive DNA synthesis between the A45Tg5P-G74DTrxA complex and the WTg5P-G74DTrxA complex could be detected.

DISCUSSION

Previously, we found that a G74D thioredoxin mutant of E. coli was unable to support the growth of WT T7 phage, and we have used this mutant strain to select for compensatory extragenic suppressors in T7 gene 5 protein (1). We found that the G74D thioredoxin mutant was compensated in vivo by six different alterations in gene 5 protein, each of which individu-
ally was both necessary and sufficient for suppression of the thioredoxin defect. Three of the suppressor mutations (V3I, ally was both necessary and sufficient for suppression of the thioredoxin defect. Three of the suppressor mutations (V3I, V32A, and A45T) were located within the 3' to 5' exonuclease domain of gene 5 protein, and the remaining three (E319V, E319K, Y409C) were within the polymerization domain of gene 5 protein.

In this study, we have extended our initial genetic analysis of the gene 5 protein-thioredoxin interaction with a biochemical analysis of the same interaction. Specifically, we have overexpressed and purified the G74D mutant thioredoxin, as well as two suppressor gene 5 proteins (E319K and A45T). These mutant gene 5 proteins were chosen to represent both classes of suppressor mutations, one (E319K) from the polymerization domain of gene 5 protein and one (A45T) from the exonuclease domain. Using the purified proteins, we have examined their interactions, and based upon our results, we have constructed a model of the gene 5 protein-thioredoxin interaction.

We believe that the E319K suppressor mutation in T7 gene 5 protein compensates for the G74D thioredoxin alteration primarily by restoring the binding affinity. We propose this mechanism of suppression for several reasons. First, the data presented in Table III indicate that the E319K mutation restored the binding affinity to the G74D thioredoxin by approximately 80%. Second, the data presented in Table IV show that the E319K mutation in gene 5 protein does not cause a significant alteration in the 3' to 5' exonuclease activity. Third, lanes 13-15 of Fig. 2 indicate that the E319K gene 5 protein is able to interact with the G74D thioredoxin to form an efficient DNA polymerase. Fourth, the burst size studies (Table V) indicate that T7-5-E319K grows quite well within the E. coli host harboring the G74D thioredoxin mutation.

Based upon these data, we conjecture that the Glu-319 residue of gene 5 protein and the Gly-74 residue of thioredoxin define a contact point between the two proteins. Specifically, we suggest that, during phage T7 DNA replication in vivo, the side chain atoms of glutamate 319 of T7 gene 5 protein are physically adjacent to the side chain hydrogen atom of glycine 74 of thioredoxin. When the Gly-74 of thioredoxin is replaced by an aspartic acid residue, the juxtaposition of the negatively charged carboxyl groups of Glu-319 (g5P) and of Asp-74 (TrxA) generates ionic repulsion, resulting in the inability of the two proteins to associate productively. When the Glu-319 of gene 5 protein is substituted with a positively charged lysine residue, repulsion due to the physical proximity of two negative charges is removed and perhaps is replaced by ionic attraction, restoring the ability to associate productively with the Asp-74 thioredoxin.

In contrast to the E319K mutation and as evident from inspection of Table III, the mechanism of suppression for the A45T mutation within gene 5 protein does not involve a direct restoration of binding affinity. The A45T mutation, located within the putative 3' to 5' exonuclease domain of gene 5 protein, did reduce the exonucleolytic activity of the protein (Table IV); however, the reduction in exonucleolytic activity was not accompanied by a detectable increase in the processivity of polymerization (Fig. 2). The results of the in vivo burst size studies (Table V) are consistent with the in vitro results, since the strain T7-5-A45T grew very poorly within E. coli cells harboring the G74D thioredoxin defect. Based on these results, we suggest that the alanine 45 residue of T7 gene 5 protein is not physically adjacent to the glycine 74 residue of thioredoxin during T7 DNA replication within infected cells. At present, we are unable to identify the nature of this compensating effect, but it could be due to the interaction of the A45Tg5P-G74DTrxA complex with other proteins at the T7 replication fork.

Based upon these functional studies, we have attempted to build a model of the gene 5 protein-thioredoxin interaction. Necessarily, the focus of this model revolves around the identification of regions or surfaces of contact between the two proteins. Fortunately, we were aided greatly in developing our model by the work of others. Dyson et al. (41) determined by nuclear magnetic resonance spectroscopy the solution structure of reduced thioredoxin, the conformation of thioredoxin that binds productively to T7 gene 5 protein (43). Eklund et al. (44) had suggested previously that a flat, hydrophobic surface surrounding the active site cysteines (Cys-32 and Cys-35) of thioredoxin plays a particularly significant role in interactions with other proteins. Russel and Model (45), investigating the requirement for E. coli thioredoxin in the assembly of the filamentous bacteriophage f1, mutagenized the entire thioredoxin gene by nitrosoguanidine and isolated three missense mutations within thioredoxin (G74D, G92S, and G92D) that caused an inability to support growth of WT f1 phage (19). Supporting the hypothesis put forth by Eklund et al. (44), both the Gly-74 and Gly-92 residues are located within the flat, hydrophobic, putative interacting surface. As previously established (1), a G92D thioredoxin mutant of E. coli does not support the growth of WT T7 phage, and in contrast to phage f1, a G92S thioredoxin mutant is able to support WT T7 growth (data not shown). Krause and Holmgren (46) found that replacing the highly conserved Trp-31 residue (adjacent to the active site Cys-32) of thioredoxin with an alanine or a histidine residue greatly reduced productive interaction with WT T7 gene 5 protein. Minarik et al. (47) found that an E. coli thioredoxin mutant strain that replaced the conserved Gly-33 residue with a tryptophan residue was unable to support the growth of WT T7 phage.

Other investigators (43, 48) have also identified this hydrophobic surface of thioredoxin (residues 31–35, 74–77, and 90–93) as critical for interactions with T7 gene 5 protein. In particular, Adler and Modrich (43) found that alkylation of free reduced thioredoxin at Cys-32 and Cys-35 by exposure to chemicals that preferentially alter sulfhydryl moieties prevented the ability of thioredoxin to stimulate the DNA polymerase activity of T7 gene 5 protein. Furthermore, T7 gene 5 protein, when associated with thioredoxin prior to exposure to the same alkylating agents, protected the inactivation of thioredoxin. Finally, other investigators have determined the solution structure of the reduced and oxidized thioredoxin and have compared the two structures (49, 50). The two forms of thioredoxin are extremely similar, essentially identical in backbone structure. The authors further suggest that the functional differences could perhaps be explained by differences in the conformational flexibility of the region surrounding the active site, especially residues 73–76. In reduced thioredoxin, the active site region was found to be more dynamic or flexible than the same region in oxidized thioredoxin. Presumably, this increased conformational flexibility in the hydrophobic region surrounding the active site facilitates interactions with other proteins.
hydrophobic region first emphasized by Eklund et al. (44), defines a surface of thioredoxin that interacts with T7 gene 5 protein. In this figure, the hydrogen atoms of the molecule are represented by its backbone only, without the side-chain atoms. Alterations in any of these five amino acid residues can cause a reduction in the ability to interact productively with T7 gene 5 protein (1, 43, 46). In this figure, nitrogen atoms are purple, and carbon atoms are gray. Oxygen atoms are red, and sulfur atoms are yellow. The positions of six amino acid residues are shown in three different colors. Phe-762 (F762), very near the site of DNA polymerization (63), is green. Based upon the homology between E. coli DNA polymerase I and T7 gene 5 protein (51, 52, 54, 64), tertiary structure inference was used to map or to position T7 gene 5 protein residues on the corresponding homologous residues of E. coli DNA polymerase I. Four amino acid residues, indicating the positions of four different T7 gene 5 suppressor mutations that compensate for the G74D thioredoxin defect (1), are colored cyan. These four amino acids are labeled with the E. coli DNA polymerase I residue followed by the corresponding T7 gene 5 protein residue, with a slash between the two residues. The suppressor or compensatory substitutions are also indicated in the label. For example, the label L404/A45T identifies this particular residue as leucine 404 of E. coli DNA polymerase I, and this residue corresponds to alanine 45 of T7 gene 5 protein, which, when substituted with threonine, compensates in vivo for the G74D thioredoxin mutation. The His-571 residue is red, and the putative thioredoxin binding domain of T7 gene 5 protein would be inserted close to or at this residue of E. coli DNA polymerase I.

Unlike E. coli thioredoxin, the structure of T7 gene 5 protein has not been determined. However, T7 gene 5 protein belongs to a family of DNA-dependent DNA polymerases that shares substantial amino acid sequence homology to E. coli DNA polymerase I (51, 52), and fortunately, the structure of the large Klenow fragment of E. coli DNA polymerase I has been determined by x-ray crystallography (53). More recently, the structure of the Klenow fragment of E. coli DNA polymerase I complexed to duplex DNA has also been determined (42). By tertiary structure inference based upon sequence homology, we propose to use the crystal structure of DNA polymerase I Klenow fragment complexed to double-stranded DNA as a rudimentary model for T7 gene 5 protein bound to duplex DNA. By this homology-based inference, we were able to match the A45T suppressor mutation in T7 gene 5 protein with its homologous counterpart in E. coli DNA polymerase I, Leu-404 (54). This is illustrated in Fig. 4.

Attempts to similarly match the Glu-319 residue of T7 gene 5 protein with the homologous residue of E. coli DNA polymerase I were not so straightforward, because, amid the highly homologous sequences, there is a domain of T7 gene 5 protein (roughly 65 amino acid residues in length) that does not have a homologous counterpart in E. coli DNA polymerase I (52, 55). The Glu-319 residue of T7 gene 5 protein is located within this domain. Previously, we had isolated 10 mutant strains of T7 that could grow within the G74D thioredoxin mutant of E. coli. Six of the suppressor mutations within gene 5 protein were located at Glu-319 (1), and the positions of the other four suppressor mutations are shown in Fig. 4. The six suppressor mutations that were located at Glu-319 altered the glutamate residue to either a lysine or a valine residue, and in this work, we have argued that the Glu-319 residue of gene 5 protein and the Gly-74 residue of thioredoxin define a contact point between the two proteins. Similarly, we have postulated that the Ala-45 residue of gene 5 protein does not contact the Gly-74 residue of thioredoxin. We now propose that the roughly 65 amino acids of T7 gene 5 protein (residues 260–325) encode a putative thioredoxin binding domain. We postulate that most of the gene 5 protein residues that contact thioredoxin directly would be located within this domain, and we estimate that, in comparison to the Klenow fragment, this added domain would be inserted into the protein sequence of E. coli DNA polymerase I somewhere close to the His-571 residue (Fig. 4, red).

In the model shown in Fig. 5, we have combined the flat, hydrophobic surface of reduced thioredoxin with the putative thioredoxin binding domain of gene 5 protein to generate a structural model of the T7 gene 5 protein-thioredoxin complex bound to duplex DNA. Our model is rudimentary and lacks the necessary details that would be required to understand precisely how thioredoxin stabilizes the primer-template-DNA polymerase complex (14). Most likely, by an unknown mechanism, thioredoxin confers processivity by physically obstructing the otherwise rapid dissociation of the newly synthesized, double-stranded DNA from T7 gene 5 protein.

Our structural model now serves as a testable, working hypothesis. Several experiments have been performed in an attempt to test this model.
newly synthesized duplex DNA and not the single-stranded template strand during DNA synthesis. Recent work supports both of these predictions.\(^5\) Perhaps the ultimate test of our structural model must await the structural determination by x-ray crystallography of the T7 gene 5 protein-thioredoxin complex bound to double-stranded DNA.

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