Interactions of Gene 2.5 Protein and DNA Polymerase of Bacteriophage T7*

(Received for publication, November 20, 1991)

Young Tae Kim‡, Stanley Tabor‡, Jorge E. Churchich§, and Charles C. Richardson‡‡

From the ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the §Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996

Bacteriophage T7 gene 2.5 protein has been shown to interact with T7 DNA polymerase (the complex of T7 gene 5 protein and Escherichia coli thioredoxin) by affinity chromatography and fluorescence emission anisotropy. T7 DNA polymerase binds specifically to a resin coupled to gene 2.5 protein and elutes from the resin when the ionic strength of the buffer is raised to 250 mM NaCl. In contrast, T7 gene 5 protein alone binds more weakly to gene 2.5 protein, eluting when the ionic strength of the buffer is 50 mM NaCl. Thioredoxin does not bind to gene 2.5 protein. Steady-state fluorescence emission anisotropy gives a dissociation constant of $1.1 \pm 0.2 \mu M$ for the complex of gene 2.5 protein and T7 DNA polymerase, with a ratio of gene 2.5 protein to T7 DNA polymerase in the complex of 1:1. Nanosecond emission anisotropic analysis suggests that the complex contains one monomer each of gene 2.5 protein, gene 5 protein, and thioredoxin. The ability of T7 gene 2.5 protein to stimulate the activity and processivity of T7 DNA polymerase is compared with the ability of three other single-stranded DNA-binding proteins: E. coli single-stranded DNA-binding protein, T4 gene 32 protein, and E. coli recA protein. All except E. coli recA protein stimulate the activity and processivity of T7 DNA polymerase; E. coli recA protein inhibits these activities.

Three proteins, T7 gene 5 protein, T7 helicase/primase, and Escherichia coli thioredoxin, account for the fundamental reactions that occur at the replication fork of bacteriophage T7 (for review, see Richardson, 1983). The 80-kDa gene 5 protein is found in phage-infected cells in a one-to-one complex with the 12-kDa thioredoxin specified by the trxA gene of E. coli (Gripp and Richardson, 1971; Oey et al., 1971; Modrich and Richardson, 1975a, 1975b; Mark and Richardson, 1976). In the absence of thioredoxin, T7 gene 5 protein is a DNA polymerase with low processivity, dissociating from a primer-template after the incorporation of only one to several nucleotides (Tabor et al., 1987; Huber et al., 1987). Thioredoxin increases the stability of the gene 5 protein–primer-template complex, conferring a high processivity to the polymerization reaction (Tabor et al., 1987; Huber et al., 1987). In this paper, for convenience, we refer to the gene 5 protein-thioredoxin complex as “T7 DNA polymerase.” T7 helicase/primase is encoded by the gene 4 protein (Dunn and Studier, 1983). Gene 4 encodes two polypeptides of molecular weight 63,000 and 56,000, the latter resulting from an internal start site in the same frame as the former. The 63-kDa gene 4 protein has both primase and helicase activity, whereas the 56-kDa protein has helicase activity but is devoid of primase activity (Bernstein and Richardson, 1988, 1989).

T7 gene 2.5 protein plays an important role in T7 DNA replication, recombination, and repair (Reuben and Gefter, 1973, 1974; Scherzinger et al., 1973; Araki and Ogawa, 1981a; Nakai and Richardson, 1988). It was originally purified based on its strong affinity for single-stranded DNA and its ability to stimulate RNA synthesis by T7 DNA polymerase (Reuben and Gefter, 1973; Scherzinger et al., 1973). Since gene 2.5 protein does not stimulate E. coli DNA polymerases I, II, or III or phage T4 DNA polymerase, it has been inferred to interact specifically with T7 DNA polymerase (Reuben and Gefter, 1973). Gene 2.5 protein has also been inferred to interact with the T7 primase, in that it stimulates the synthesis of RNA primers catalyzed by T7 primase and increases the efficiency with which these primers are used by T7 DNA polymerase to initiate lagging strand synthesis (Nakai and Richardson, 1988).

Both E. coli single-stranded DNA-binding (SSB) protein and T7 gene 2.5 protein stimulate DNA synthesis by T7 DNA polymerase on primed, single-stranded DNA (Reuben and Gefter, 1973, 1974; Scherzinger et al., 1973; Tabor et al., 1987). In the case of E. coli SSB protein, this stimulation results from its ability to increase the processivity of T7 DNA polymerase, presumably by removing secondary structure impediments in the DNA (Tabor et al., 1987). Although the mechanism of the stimulation of T7 DNA polymerase by gene 2.5 protein has not been determined, it is reasonable to speculate that it occurs by a similar mechanism. Genetic evidence suggests that this function of gene 2.5 protein can be replaced by its E. coli counterpart: a T7 mutant with an amber lesion in gene 2.5 which expresses a shortened polypeptide approximately 90% the length of wild-type gene 2.5 protein can grow on E. coli strains expressing a functional SSB protein but not on strains expressing a temperature-sensitive SSB protein at the nonpermissive temperature (Araki and Ogawa, 1981b). On the other hand, other mutations in gene 2.5 suggest that at least a partially functional gene 2.5 protein is essential

* This investigation was supported by United States Public Health Service Grant AI-06045, Department of Energy Grant DE-FG02-88ER60688, and Grant NP-IT from the American Cancer Society Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be sent: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1864; Fax: 617-432-3062.

‡§ The abbreviations used are: SSB, single-stranded DNA binding; BSA, bovine serum albumin; FITC, fluoresceine isothiocyanate; EDANS, N-iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid; DTT, dithiothreitol; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; SED, standard enzyme diluent.
even in wild type E. coli strains. In this paper, we compare the effect of four proteins that bind to single-stranded DNA, T7' gene 2.5 protein, E. coli SSB protein, T4 gene 32 protein, and E. coli recA protein, on the processivity of T7 DNA polymerase.

In its role in DNA replication, it is likely that gene 2.5 protein interacts with T7 DNA polymerase. For this reason we have, in the present study, examined their interaction directly using two different techniques, affinity chromatography and fluorescence emission anisotropy. Affinity chromatography is a useful technique for demonstrating protein-protein interactions. For example, it was used by Formosa et al. (1983) to demonstrate an interaction between T4 gene 32 protein and three proteins: T4 DNA polymerase (gene 43 protein) and two major proteins in the recombination pathway (T4 uvuX and uvuY proteins). Fluorescence emission anisotropy is a powerful tool for studying the interaction between proteins (for review, see Lakowicz, 1983). In this method, a fluorophore is attached to one of the proteins, and the extent of polarization, or anisotropy, of the fluorescence emitted from that fluorophore is measured in the presence of increasing amounts of the second protein. Anisotropy is a measure of the degree of rotation of the fluorophore which occurs between absorption and emission of the photon, a value that will vary depending on the size of the complex bound to the fluorophore. Thus the anisotropy of a protein will increase when it complexes with other proteins. Anisotropic measurements can determine accurately the stoichiometry of protein complexes and their dissociation constants. As an example, this technique has been used successfully to study the interactions between pyridoxal kinase and aspartate aminotransferase (Kim et al., 1988). In this study we use it to study the interaction of gene 2.5 protein and T7 DNA polymerase.

EXPERIMENTAL PROCEDURES

Materials

Proteins—Gene 2.5 protein was purified to apparent homogeneity from cells overexpressing its gene as described in the accompanying paper (Kim et al., 1992). T7 DNA polymerase (T7 gene 5 protein and E. coli thioredoxin complex) was purified from cells overproducing both polypeptides (Tabor et al., 1987). T7 gene 5 protein was overproduced and purified to homogeneity from an E. coli strain deficient in trxA, the gene for thioredoxin (Tabor et al., 1987). T7 RNA polymerase was purified from cells overexpressing its gene as described by Tabor and Richardson (1985). T7 gene 4 protein consisting of the 56-kDa and 63-kDa forms in equimolar amounts was purified as described (Nakai and Richardson, 1988). The 56-kDa form of T7 gene 4 protein was purified in the absence of the 63-kDa form as described (Bernstein and Richardson, 1988). E. coli thioredoxin was overproduced in cells containing a clone of its gene (Tabor et al., 1986) and purified according to the procedure of Modrich and Richardson (1976b). E. coli SSB protein, E. coli recA protein, T4 gene 32 protein, and T4 polynucleotide kinase were obtained from U. S. Biochemical Corp. Bovine serum albumin (BSA) was from Miles Laboratories.

DNA and Nucleotides—Single-stranded M13mp18 DNA was prepared as described (Tabor et al., 1997). The 17-mer "20" M13 primer (GTAAACGACGGCCAGT) was provided by A. Nusshaum (Harvard Medical School). Alkaline phosphatase (T7 gene 6 protein) and affinity-chromatography coupled to the plane of incident polarized light. Affinity HNB sheet polarizers were used for excitation and emission anisotropy (F0(t)) and perpendicular (F90(t)) to the plane of incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission anisotropy measurements containing either FITC- or EDANS-labeled gene 2.5 protein and (where indicated) unlabeled T7 DNA polymerase in buffer B containing 50 mM KCl. A constant temperature of 25 °C was maintained using a thermostated cell.

Emission anisotropies (steady-state fluorescence decay measurements) were recorded on an SLM double-beam fluorescence apparatus equipped with a RACAL-DANAN digital multimeter. Illumination was achieved by a Xenon lamp (200 watts) and passed through a grating monochromator (Bausch and Lomb). The emission was filtered through a Corning C-3-49 glass filter. Emission anisotropies were measured with a precision of ±0.002.

Fluorescence decay measurements were made using the monochromatic technique with an Ortec model 8200 nanosecond spectrometer. A fixed running flash lamp operating in air at 1 atm was used as the excitation source. The lamp was pulsed at 10 kHz. Excitation was at 340 nm, and the emission was filtered through a Corning C-3-49 glass filter. Rotational correlation times were determined using the fluorescence decay curves of the polarized components parallel (F0(t)) and perpendicular (F90(t)) to the plane of incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission anisotropy measurements containing either FITC- or EDANS-labeled gene 2.5 protein and (where indicated) unlabeled T7 DNA polymerase in buffer B containing 50 mM KCl. A constant temperature of 25 °C was maintained using a thermostated cell.

Emission anisotropies (steady-state fluorescence decay measurements) were recorded on an SLM double-beam fluorescence apparatus equipped with a RACAL-DANAN digital multimeter. Illumination was achieved by a Xenon lamp (200 watts) and passed through a grating monochromator (Bausch and Lomb). The emission was filtered through a Corning C-3-49 glass filter. Emission anisotropies were measured with a precision of ±0.002.

Fluorescence decay measurements were made using the monochromatic technique with an Ortec model 8200 nanosecond spectrometer. A fixed running flash lamp operating in air at 1 atm was used as the excitation source. The lamp was pulsed at 10 kHz. Excitation was at 340 nm, and the emission was filtered through a Corning C-3-49 glass filter. Rotational correlation times were determined using the fluorescence decay curves of the polarized components parallel (F0(t)) and perpendicular (F90(t)) to the plane of incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission anisotropy measurements containing either FITC- or EDANS-labeled gene 2.5 protein and (where indicated) unlabeled T7 DNA polymerase in buffer B containing 50 mM KCl. A constant temperature of 25 °C was maintained using a thermostated cell.

Emission anisotropies (steady-state fluorescence decay measurements) were recorded on an SLM double-beam fluorescence apparatus equipped with a RACAL-DANAN digital multimeter. Illumination was achieved by a Xenon lamp (200 watts) and passed through a grating monochromator (Bausch and Lomb). The emission was filtered through a Corning C-3-49 glass filter. Emission anisotropies were measured with a precision of ±0.002.

Fluorescence decay measurements were made using the monochromatic technique with an Ortec model 8200 nanosecond spectrometer. A fixed running flash lamp operating in air at 1 atm was used as the excitation source. The lamp was pulsed at 10 kHz. Excitation was at 340 nm, and the emission was filtered through a Corning C-3-49 glass filter. Rotational correlation times were determined using the fluorescence decay curves of the polarized components parallel (F0(t)) and perpendicular (F90(t)) to the plane of incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission anisotropy measurements containing either FITC- or EDANS-labeled gene 2.5 protein and (where indicated) unlabeled T7 DNA polymerase in buffer B containing 50 mM KCl. A constant temperature of 25 °C was maintained using a thermostated cell.

Emission anisotropies (steady-state fluorescence decay measurements) were recorded on an SLM double-beam fluorescence apparatus equipped with a RACAL-DANAN digital multimeter. Illumination was achieved by a Xenon lamp (200 watts) and passed through a grating monochromator (Bausch and Lomb). The emission was filtered through a Corning C-3-49 glass filter. Emission anisotropies were measured with a precision of ±0.002.

Fluorescence decay measurements were made using the monochromatic technique with an Ortec model 8200 nanosecond spectrometer. A fixed running flash lamp operating in air at 1 atm was used as the excitation source. The lamp was pulsed at 10 kHz. Excitation was at 340 nm, and the emission was filtered through a Corning C-3-49 glass filter. Rotational correlation times were determined using the fluorescence decay curves of the polarized components parallel (F0(t)) and perpendicular (F90(t)) to the plane of incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission anisotropy measurements containing either FITC- or EDANS-labeled gene 2.5 protein and (where indicated) unlabeled T7 DNA polymerase in buffer B containing 50 mM KCl. A constant temperature of 25 °C was maintained using a thermostated cell.
emission. Anisotropic decay analysis was performed using the method of the "sum and difference" (O'Connor and Phillips, 1984). The function
\[ D(t) = F(t) - F(0) \]
was deconvoluted and analyzed by a nonlinear least square method (Ware et al., 1973). The deconvoluted curves were fitted to Equation 1
\[ F(t) - F(0) = 3 \Delta e^{-\gamma \alpha t^3} \]
where \( \tau \) is the fluorescence decay time of the function \( F(t) = F_0(t) + 2 F(t) + \text{measured in a separate experiment} \), and \( \phi \) is the rotational correlation time. The adequacy of the exponential decay fitting was judged by inspection of the weighted residual plots and reduced \( \chi^2 \) (chi-squared).

Fluorescence spectra were recorded in a precision spectrophotometer equipped with two Bausch and Lomb monochromators. Absorption spectra were recorded with a Shimadzu (model UV-16) and Hewlett-Packard (model 8452A) spectrophotometer.

**DNA Polymerase Activity Assay**—The DNA polymerase assay was a modification of that described by Tabor and Richardson (1989). The 17-mer primer was annealed to single-stranded M13mp19-2 DNA in a reaction mixture (10 \( \mu \)l) containing 2 \( \mu \)g of M13mp19-2 DNA, 6 ng of primer (a 2-fold molar excess to template), 40 \( \mu \)M Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), and 50 mM NaCl. The mixture was heated to 65 °C for 2 min and then cooled to room temperature over 30 min. For the processivity reaction mixture for the DNA polymerase assay (40 \( \mu \)l) contained 2 \( \mu \)g of primed M13mp19-2 DNA; 50 \( \mu \)M Tris-HCl, pH 7.5; 12.5 mM MgCl\(_2\); 6.25 mM DTT; 25 mM NaCl; and a 0.375 mM concentration of dGTP, dATP, dCTP, and [\(^3\)H]TTP (30 cpn/pmol).

To the above mixture was added 5 \( \mu \)l of 40 \( \mu \)M Tris-HCl, pH 7.5, 5 mM mercaptoethanol, and 0.5 mg/ml BSA (standard enzyme diluent or SED) or 5 \( \mu \)l of the indicated amount of gene 2.5 protein. Each reaction mixture for the processivity assay (40 \( \mu \)l) contained 5 \( \mu \)l of SED containing 27 ng (0.3 pmol) of T7 DNA polymerase. The reaction was stopped by the addition of 5 \( \mu \)l of 200 mM EDTA.

**Fluorescence**
Fluorescence spectra were recorded with a Shimadzu (model UV-16) and Hewlett-Packard (model 8452A) spectrophotometer.

**Absorption**
Absorption spectra were recorded with a Shimadzu (model UV-16) and Hewlett-Packard (model 8452A) spectrophotometer.

**RESULTS**

**Gene 2.5 Protein Affinity Chromatography**—To determine whether T7 DNA polymerase could interact directly with gene 2.5 protein, we examined its ability to bind to gene 2.5 protein covalently coupled to a resin. Gene 2.5 protein was coupled to Affi-Gel at a concentration of 4 mg of gene 2.5 protein/ml of resin. The resin (0.25 ml) was mixed with 0.4 mg of T7 DNA polymerase and after incubating for 20 min at 4 °C, was washed with increasing concentrations of NaCl. In the absence of NaCl, greater than 90% of the T7 DNA polymerase bound to the resin (Fig. 1A). Less than 5% of the T7 DNA polymerase eluted with NaCl concentrations of 50 and 150 mM. However, upon the addition of 250 mM NaCl, ~90% of the bound T7 DNA polymerase eluted from the column.

As a control, the identical experiment was carried out using T7 DNA polymerase using Affi-Gel that had been coupled with BSA (Fig. 1B). In this case, most of the T7 DNA polymerase eluted in the absence of NaCl, with only a low level (less than 5%) binding to the column. Thus the affinity of T7 DNA polymerase for the resin coupled to gene 2.5 protein is a result of an interaction of T7 DNA polymerase with gene 2.5 protein and not of any nonspecific interactions between the polymerase and the resin.

In the experiments described above, the form of the T7 DNA polymerase applied to the column was a one-to-one complex of T7 gene 5 protein and E. coli thioredoxin (Tabor et al., 1987). Since gene 5 protein in the absence of thioredoxin

**FIG. 1. Gene 2.5 protein affinity chromatography of T7 DNA polymerase.** T7 DNA polymerase (T7 gene 5 protein and E. coli thioredoxin complex) was applied to Affi-Gel resin that had been coupled with either gene 2.5 protein (A) or BSA (B) as described under "Experimental Procedures." T7 DNA polymerase was eluted from the column using 1-mI step gradients containing 0, 50, 150, 250, and 500 mM NaCl (arrows). 200-\( \mu \)l fractions were collected, the absorbances at 280 nm were determined (0), and aliquots were assayed for DNA polymerase activity (O).
is a DNA polymerase, with the role of thioredoxin being to confer processivity on the polymerase reaction (see "Introduction"); it was of interest to determine the affinity of gene 2.5 protein for each protein separately (Fig. 2, A and B). The interaction of gene 2.5 protein with gene 5 protein is weaker than that observed with the gene 5 protein-thioredoxin complex, the gene 5 protein eluting from the column with 50 mM NaCl (Fig. 2C). Thioredoxin does not bind to the column even in the absence of NaCl (Fig. 2B). These data suggest that although the principal contacts between gene 2.5 protein and T7 DNA polymerase are with the gene 5 protein, the presence of thioredoxin alters the conformation of the gene 5 protein such that it binds to gene 2.5 protein more tightly.

We also used affinity chromatography to determine whether two other proteins involved in T7 DNA replication, T7 RNA polymerase (gene 1 protein) and helicase/primase (gene 4 protein), interact with the gene 2.5 protein. T7 RNA polymerase did not bind to the Affi-Gel resin coupled to gene 2.5 protein even in the absence of NaCl, suggesting that it does not interact with gene 2.5 protein (Fig. 2C). On the other hand, the helicase/primase (an equimolar ratio of the 56-kDa and 63-kDa peptides; see Nakai and Richardson, 1988) bound to the column in the absence of NaCl and in the presence of 50 mM NaCl, and both peptides eluted together from the column in the presence of 150 mM NaCl. Neither peptide was retained by the Affi-Gel resin coupled with BSA. A preparation consisting solely of 56-kDa gene 4 protein, which has helicase activity but lacks primase activity (Bernstein and Richardson, 1988), also bound to the Affi-Gel resin coupled with gene 2.5 protein and eluted at the same NaCl concentration (150 mM) as the mixture. Thus T7 helicase/primase interacts with gene 2.5 protein, albeit with a weaker affinity than the interaction between gene 2.5 protein and T7 DNA polymerase (gene 5 protein-thioredoxin complex).

Fluorescence Emission Anisotropic Measurements of the Interaction between T7 Gene 2.5 Protein and DNA Polymerase—The results of affinity chromatography show that T7 DNA polymerase and gene 2.5 protein interact with a relatively high affinity. However, it is difficult using affinity chromatography to obtain information on the stoichiometry of the complex or on the binding constant of the interaction. Fluorescence emission anisotropy, on the other hand, gives precise measurements on protein-protein interactions (see Lakowicz, 1983). In this technique, one of the proteins is fluorescently labeled, and the polarization, or anisotropy, of the fluorophore is measured in the presence and absence of the second, unlabeled protein species. We describe here the labeling of gene 2.5 protein separately with two fluorophores, FITC and EDANS, and measurements of the anisotropy in the presence and absence of T7 DNA polymerase.

Gene 2.5 protein was labeled with FITC to measure the steady-state emission anisotropy. We used reaction conditions that modify selectively the amino terminus of proteins (Haugland, 1989). The average of incorporation was 0.9 FITC molecules/molecule of gene 2.5 protein monomer. The reaction of gene 2.5 protein with FITC had no effect on the ability of gene 2.5 protein to stimulate the DNA polymerase activity of T7 DNA polymerase (data not shown), suggesting that it did not affect significantly the interaction between the two proteins. The [FITC]gene 2.5 protein complex was excited at 460 nm, and the emission steady-state anisotropy was determined in the presence of increasing amounts of T7 DNA polymerase.

When [FITC]gene 2.5 protein is the only component of the system, the emission anisotropy ($A_0$) is determined by the Brownian rotation of the tagged macromolecule (Lakowicz, 1983). Upon the addition of T7 DNA polymerase, a progressive increase in the emission anisotropic values was observed (Fig. 3). This increase is caused by the formation of [FITC] gene 2.5 protein-T7 DNA polymerase complex, as indicated in Equation 2, which depicts an equilibrium between free and bound proteins. The Brownian rotation of the tagged macromolecule is restricted upon formation of a complex with the DNA polymerase; consequently correlation between concentration of complex formed and the emission anisotropy is observed.

\[
[\text{FITC}]\text{Gene 2.5 protein} + \text{T7 DNA polymerase} \rightleftharpoons [\text{FITC}]\text{gene 2.5 protein} \; \text{DNA polymerase}
\]
The increase in emission anisotropy by the addition of T7 DNA polymerase was detected when the binding was carried out under conditions of low ionic strength (50 mM KCl in Fig. 3). As a control, the experiment was also carried out in 400 mM KCl, an ionic strength that leads to dissociation of gene 2.5 protein and T7 DNA polymerase (Fig. 1A); under these conditions T7 DNA polymerase does not increase the anisotropy (Fig. 3). This confirms that the anisotropic effects observed at low ionic strength are a result of an interaction between the two proteins.

In the presence of excess T7 DNA polymerase, the equilibrium in Equation 2 is shifted in the direction of the formation of the complex, and the emission anisotropy approaches a maximum value \( A_{\infty} \). In the experiment shown in Fig. 3, in which the concentration of [FITC] gene 2.5 protein was 3 \( \mu \)M, the concentration of T7 DNA polymerase required to obtain the maximum emission anisotropy was also 3 \( \mu \)M. Thus, the emission anisotropy of the system reaches a maximum value when the molar ratio of both proteins approaches 1.

The fraction \( \alpha \) of [FITC] gene 2.5 protein bound to T7 DNA polymerase can be calculated from Equation 3

\[
\alpha = \frac{A - A_0}{(A_0 - A_\infty)\beta + (A_\infty - A)}
\]

where \( A \) is the observed emission anisotropy of the mixture of bound and unbound [FITC] gene 2.5 protein, \( A_0 \) is the observed emission anisotropy of free [FITC] gene 2.5 protein, \( A_{\infty} \) is the maximum observed emission anisotropy of [FITC] gene 2.5 protein in the presence of a saturating amount of T7 DNA polymerase, and \( \beta \) is the ratio of fluorescence yield of bound (\( q_b \)) versus free (\( q_f \)) [FITC] gene 2.5 protein. \( \beta (\beta = 0.75) \) must be taken into consideration in the determination of \( \alpha \) since the fluorescence intensity of gene 2.5 protein labeled with either FITC or EDANS is quenched upon binding T7 DNA polymerase. This equation assumes that the anisotropy for bound gene 2.5 protein is the same for all possible complexes. Using this technique it is virtually impossible to detect intermediates containing 2:1 or 1:1 molecules. We can measure only an average value corresponding to several species.

We carried out anisotropic measurements of gene 2.5 protein in the presence of T7 DNA polymerase to determine the affinity of the two proteins. First, the anisotropy was measured using a constant amount of [FITC] gene 2.5 protein and a varying concentration of T7 DNA polymerase (Fig. 4). The dissociation constant \( K_d \) of [FITC] gene 2.5 protein and T7 DNA polymerase was determined by Equation 4

\[
\frac{1}{\alpha} = 1 + \frac{K_d}{[P]}
\]

where \( P \) is the concentration of free T7 DNA polymerase, and \( \alpha \), the fraction of [FITC] gene 2.5 protein bound to T7 DNA polymerase, was obtained from Equation 3. In Fig. 4, 1/\( \alpha \) has been plotted as a function of 1/[P]. From these data, a dissociation constant of 1.03 \( \mu \)M is calculated. We have also analyzed the binding data using Enzfitter program (R. J. Leatherbarrow) and found the number of binding sites is equal to one; no cooperativity in the binding process was detected. An apparent dissociation constant of gene 2.5 protein and T7 DNA polymerase was also determined by measuring the anisotropy \( A_\infty \) of serial dilutions of the [FITC] gene 2.5 protein-T7 DNA polymerase complex (Fig. 5). The anisotropy reaches half-maximum value at 1.1 \( \mu \)M gene 2.5 protein, in close agreement to the binding constant determined from Equation 4.

**Nanosecond Emission Anisotropy**—The steady-state fluorescence measurements using FITC indicate a moderately tight complex between gene 2.5 protein and T7 DNA polymerase. To obtain information on the size and degree of flexibility of the complex, we carried out nanosecond emission anisotropy by time correlated single-photon counting (O'Connor and Phillips, 1984).

Nanosecond emission anisotropy requires that the fluorophore have a decay time of sufficient duration to measure the rotational correlation time of the complex. ~40 ns for a complex of gene 2.5 protein and T7 DNA polymerase (see "Discussion"). Protein-bound EDANS, with a decay time of 19 ns, is better suited as the fluorophore for use in this technique than protein-bound FITC, with a decay time of 5 ns (O'Connor and Phillips, 1984). EDANS binds covalently.
to cysteine residues. Gene 2.5 protein has three cysteine residues (Dunn and Studier, 1983). EDANS was reacted with gene 2.5 protein to an extent that 1.1 mol of EDANS were covalently bound per mol of gene 2.5 protein. This degree of modification did not affect the ability of gene 2.5 protein to stimulate the DNA polymerase activity of T7 DNA polymerase (data not shown).

Prior to carrying out time-dependent anisotropic measurements, we first carried out steady-state fluorescence measurements on the [EDANS]gene 2.5 protein-T7 DNA polymerase complex analogous to those just described using FITC. As was the case with [FITC]gene 2.5 protein, the addition of T7 DNA polymerase to [EDANS]gene 2.5 protein resulted in fluorescence quenching (Fig. 6A). The effect of the concentration of T7 DNA polymerase on the extent of fluorescence quenching and the increase in anisotropy is shown in Fig. 6, B and C, respectively. These results indicate an equimolar ratio of gene 2.5 protein and T7 DNA polymerase in the complex, as was the case for the [FITC]gene 2.5 protein-T7 DNA polymerase complex. The dissociation constant of the [EDANS]gene 2.5 protein and T7 DNA polymerase was 1.3 \( \mu \)M, similar to the values obtained for [FITC]gene 2.5 protein and T7 DNA polymerase (1.09–1.1 \( \mu \)M).

The results of time-dependent emission anisotropic measurements on free [EDANS]gene 2.5 protein are given in Fig. 7A and Table I, where it can be seen that the decay of the emission anisotropy is accurately described by the monoexponential decay equation.

\[ A(t) = A_0 e^{-t/\tau} \]  

Using a value of  \( A_0 = 0.24 \) for the initial anisotropy (the \( y \) intercept in Fig. 7A), one obtains a rotational correlation time of 24 ns for free [EDANS]gene 2.5 protein.

The results of the time-dependent emission anisotropic measurements on [EDANS]gene 2.5 protein in the presence of a saturating amount of T7 DNA polymerase are shown in Fig. 7B and Table I. Fitting the decay function \( A(t) \) to the monoeponential decay Equation 5 using a value of  \( A_0 = 0.29 \) (the \( y \) intercept in Fig. 7B), a rotational correlation time of 42 ns is obtained for the gene 2.5 protein-T7 DNA polymerase complex. From these results, it is clear that the rotational motion of the gene 2.5 protein is restricted upon binding to T7 DNA polymerase. The measured correlation time is most consistent with the formation of a one-to-one complex of gene 2.5 protein and T7 DNA polymerase (see “Discussion”).

**Stimulation of T7 DNA Polymerase Activity**—Both T7 gene 2.5 protein and E. coli SSB protein stimulate T7 DNA polymerase on single-stranded DNA (Reuben and Gefter, 1973, 1974; Scherzinger et al., 1973; Tabor et al., 1987). We compared the ability of these two proteins to stimulate T7 DNA polymerase with two other proteins that bind to single-stranded DNA, T4 gene 32 protein, and E. coli recA protein. The assay we used measured DNA synthesis on primed single-stranded M13 DNA during a 1-min incubation. Although E.

---

**Fig. 6.** Effect of T7 DNA polymerase on the steady-state fluorescence and anisotropy of [EDANS]gene 2.5 protein. A, emission spectra of [EDANS]gene 2.5 protein in the absence (1) and presence (2) of T7 DNA polymerase. The [EDANS]gene 2.5 protein concentration was 3 \( \mu \)M, and the T7 DNA polymerase concentration (when present) was 4 \( \mu \)M. The excitation wavelength was 340 nm. B, effect of T7 DNA polymerase on the fluorescence of [EDANS]gene 2.5 protein. C, effect of T7 DNA polymerase on the emission anisotropy of [EDANS]gene 2.5 protein. For B and C, the [EDANS]gene 2.5 protein concentration was 3 \( \mu \)M. The excitation wavelength was 340 nm using unpolarized light. The fluorescence intensity values (C) and emission anisotrope values (O) were measured at increasing concentrations of T7 DNA polymerase as described under “Experimental Procedures.”

**Table I**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Fluorescence lifetime (ns)</th>
<th>Rotational correlation time (( \tau ))</th>
<th>( x^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>[EDANS]gene 2.5 protein</td>
<td>19.0</td>
<td>24</td>
<td>1.20</td>
</tr>
<tr>
<td>[EDANS]gene 2.5 protein plus T7 DNA polymerase</td>
<td>18.8</td>
<td>42</td>
<td>1.23</td>
</tr>
</tbody>
</table>

* The concentrations of [EDANS]gene 2.5 protein and T7 DNA polymerase are 6 \( \mu \)M and 30 \( \mu \)M, respectively. 

\( x^2 \) measures the adequacy of the monoexponential decay fitting of the function \( A(t) \), the anisotropic decay.
coli SSB protein, T7 gene 2.5 protein, and T4 gene 32 protein all stimulate T7 DNA polymerase activity. E. coli recA protein inhibits T7 DNA polymerase activity (Fig. 8). The extent of stimulation was dependent on the temperature of the reaction and the ionic strength of the mixture; the greatest stimulation was obtained at lower temperatures and higher ionic strength, conditions that maximize the secondary structure in the single-stranded DNA (data not shown). At 20 °C, 50 mM NaCl and a weight ratio of binding protein to DNA of 7.5 to 1, there is a 60-fold stimulation by E. coli SSB protein, 12-fold stimulation by T7 gene 2.5 protein, 3-fold stimulation by T4 gene 32 protein, and a 2-fold inhibition by E. coli recA protein (Fig. 8).

Stimulation of Processivity of T7 DNA Polymerase—One mechanism by which single-stranded DNA-binding proteins can stimulate the activity of T7 DNA polymerase is by increasing the processivity of DNA synthesis (Tabor et al., 1987). We compared the effect of the four binding proteins discussed above (E. coli SSB protein, T7 gene 2.5 protein, T4 gene 32 protein, and E. coli recA protein) on the processivity of T7 DNA polymerase. Processivity was measured on a single-stranded, circular M13 DNA template to which was annealed a 5'-32P-labeled primer. The reaction conditions used were identical to that used for the DNA polymerase assay to correlate the stimulation (or inhibition) of DNA synthesis with the processivity. An excess of the primer-template was incubated with a limiting amount of T7 DNA polymerase, and the extent of DNA synthesis was determined by agarose-gel electrophoresis followed by autoradiography (Fig. 9). As we have demonstrated previously (Tabor et al., 1987), T7 DNA polymerase is moderately processive in the absence of any binding protein at 37 °C; under conditions in which most primers had not been extended, some of the primers were elongated the entire distance around the circular DNA molecule, ~10,000 nucleotides, as reflected by the radioactively labeled band migrating at the position corresponding to double-stranded, nicked DNA molecules (Fig. 9, panel A).

It is known that the processivity of T7 DNA polymerase in the absence of binding proteins is markedly temperature-dependent (Tabor et al., 1987). Reducing the temperature of the reaction from 37 to 20 °C results in a dramatic reduction in the extent of elongation of each primer (Fig. 9, panel B).

**Fig. 8.** The effect of single-stranded DNA-binding proteins on DNA synthesis by T7 DNA polymerase. The incorporation of [3H]dTTP into primed single-stranded M13 DNA was determined as described under “Experimental Procedures.” The molar ratio of primer-template molecules to T7 DNA polymerase was approximately 2:1, and the reaction was carried out at 20 °C for 60 s. Each reaction mixture contained 2 μg M13mp18 DNA and the indicated amount of E. coli SSB protein (■), T7 gene 2.5 protein (●), T4 gene 32 protein (□), or E. coli recA protein (○). The total incorporation of 4dNMPs into DNA is plotted as a function of the concentration of each DNA-binding protein.

This reflects a lower observed processivity of the polymerase and not simply a slower rate of synthesis since the extent of elongation does not change appreciably with incubations of 1–10 min (data not shown). At the lowest concentration of T7 DNA polymerase (a 10:1 ratio of primer-template to polymerase molecules), virtually no DNA synthesis could be detected at 20 °C. The effect of increasing concentrations of E. coli SSB protein, T7 gene 2.5 protein, T4 gene 32 protein, and E. coli recA protein was analyzed on the processivity of DNA synthesis using a 1:10 ratio of primer-template to polymerase molecules at 20 °C. E. coli SSB protein stimulates dramatically the processivity of T7 DNA polymerase; the addition of 3, 6, and 9 μg results in an increasing number of primers extended the entire distance around the single-stranded DNA template (Fig. 9, panel C). In the presence of E. coli SSB protein, there is an absence of sites corresponding to pausing or dissociation of the polymerase. T7 gene 2.5 protein also stimulates the processivity of T7 DNA polymerase; however, a higher concentration of gene 2.5 protein is required compared with E. coli SSB protein to obtain the same degree of stimulation, (Fig. 9, panel D). In the presence of gene 2.5 protein, the polymerase pauses or dissociates at several sites on the template. T4 gene 32 protein also stimulates the processivity of T7 DNA polymerase; however, it is clearly less effective than either E. coli SSB protein or T7 gene 2.5 protein, being required at higher levels to obtain a comparable effect (Fig. 9, panel E). Under these conditions, in which the extent of DNA synthesis by T7 DNA polymerase is virtually undetectable in the absence of any binding protein, the addition of E. coli recA protein has no detectable effect (Fig. 9, panel F). At 37 °C, where T7 DNA polymerase alone has high processivity (Fig. 9, panel A), increasing amounts of E. coli recA protein results in a decrease in the processivity of the polymerase (Fig. 9, panel G).

**Fig. 9.** The effect of single-stranded DNA-binding proteins on the processivity of T7 DNA polymerase. The primer-template was a 32P-labeled 17-mer primer annealed to the 10,000 nucleotide single-stranded circular M13mp18-2 DNA as described under “Experimental Procedures.” Each reaction mixture contained 2 μg of single-stranded DNA. The reactions were carried out at either 37 °C (panels A and G) or 20 °C (panels B–F) for 3 min. The reaction mixtures contained 3, 6, or 9 μg of either E. coli SSB protein (panel C), T7 gene 2.5 protein (panel D), T4 gene 32 protein (panel E), or E. coli recA protein (panels F and G). The ratios of primer-template to T7 DNA polymerase molecules are indicated at the bottom; in panels C–F, each reaction mixture contained 5 ng of T7 DNA polymerase, and in panel G, each reaction mixture contained 1.5 μg of T7 DNA polymerase. Samples were electrophoresed in a 0.6% agarose gel containing 0.5 μg/ml ethidium bromide. After electrophoresis the gel was dried and analyzed by autoradiography. Markers (0 and 10,000) refer to the number of nucleotides incorporated.

**DISCUSSION**

In this paper we demonstrate by affinity chromatography and anisotropic emission analysis a specific interaction between T7 gene 2.5 protein and T7 DNA polymerase. The complex is stable at moderate ionic strengths (50 mM KCl) but dissociates when the ionic strength of the medium is

---

**Gene 2.5 Protein and DNA Polymerase of Phage T7**

---

**Figure 8**

**Figure 9**

---

**Table**

---

**Graph**

---

**Protocol**

---

**Conclusion**

---

**Discussion**

---
increased above 150 mM. Emission anisotropic measurements indicate that gene 2.5 protein and T7 DNA polymerase associate with one another with an equilibrium dissociation constant of 1 \( \mu \text{M} \). These measurements were carried out independently using two different fluorophores; one (FITC) bound to the amino terminus of gene 2.5 protein and the other (EDANS) bound to a cysteine residue of gene 2.5 protein. In neither case does the coupling of gene 2.5 protein with the fluorophore reduce the ability of gene 2.5 protein to stimulate DNA polymerase by T7 DNA polymerase, arguing that the reaction of gene 2.5 protein with either fluorophore did not interfere significantly with its binding to T7 DNA polymerase. With both fluorophores there is a significant amount of quenching observed upon the binding of gene 2.5 protein to T7 DNA polymerase, indicating that each is in close proximity to the T7 DNA polymerase after binding.

The rotational correlation time of a molecule determined by single photon counting is dependent on the size of the complex coupled to the fluorophore. Labeled gene 2.5 protein has a rotational correlation time of 24 ns, which increases to 42 ns in the presence of a saturating level of T7 DNA polymerase. This clearly reflects that the labeled gene 2.5 protein is becoming rigidly trapped by T7 DNA polymerase. What is the stoichiometry of the proteins in this complex? For a spherical macromolecule, the overall correlation time can be calculated by Equation 6

\[
\phi_0 = \frac{V}{\kappa T}
\]  

where \( \eta \) is the viscosity of the solution, \( T \) is the absolute temperature (273 K), \( V \) is the volume of the rotating unit, and \( \kappa \) is Boltzmann’s constant. The volume of the rotating unit is given by Equation 7

\[
V = (v + h) \frac{M_0}{N_0}
\]  

where \( v \) is the partial specific volume for a spherical model (\( v = 0.73 \text{ cm}^3/\text{g} \)), \( h \) is the degree of hydration, \( M_0 \) is the molecular weight, and \( N_0 \) is Avogadro’s number. The value of \( h \), 0.2 g H2O/g of protein, is an average value for globular proteins.

Based on the above equations, a spherical molecule of \( M_0 = 25,600 \) would have a calculated rotational correlation time of 10 ns, and a molecule of \( M_0 = 51,100 \) would have a rotational correlation time of 20 ns. Thus the rotational correlation time of labeled gene 2.5 protein in the absence of T7 DNA polymerase, 24 ns, is close to that predicted for a spherical hydrated dimeric structure of 51 kDa. This is consistent with our demonstration by direct physical measurements that gene 2.5 protein, in the absence of other proteins or DNA, is a dimer of two identical subunits of molecular weight 25,562 (Kim et al., 1992).

If a dimer of gene 2.5 protein bound a monomer of T7 DNA polymerase (gene 5 protein-thioredoxin complex), the \( M_0 \) of 143,000 would yield a calculated rotational correlation time of 56 ns. A complex made up of a monomer of gene 2.5 protein and a monomer of T7 DNA polymerase would have a \( M_0 \) of 117,000 and a calculated rotational correlation time of 44 ns. A monomer of T7 DNA polymerase would have a \( M_0 \) of 91,000 and a calculated rotational correlation time of 35 ns. Hence, the rotational correlation time determined for the gene 2.5 protein-T7 DNA polymerase complex of 42 ns is nearly identical to that predicted for a compact sphere made up of one monomer of gene 2.5 protein and one monomer of T7 DNA polymerase. This is consistent with our determinations of the stoichiometry of binding of T7 DNA polymerase to gene 2.5 protein (Figs. 3 and 6 C), which indicates a 1:1 of gene 2.5 protein to T7 DNA polymerase in the complex of the two proteins.

It should be cautioned that the calculations made above are based on a model that does not take into account the deviations from spherical shape and changes in the degree of hydration, parameters that can affect the observed rotational correlation times. However, it is clear that the experimentally determined value for the gene 2.5 protein-T7 DNA polymerase complex reflects the rotation of a labeled gene 2.5 protein rigidly trapped by the T7 DNA polymerase.

Gene 5 protein binds to gene 2.5 protein significantly more tightly in the presence of thioredoxin than in its absence, with 250 and 50 mM NaCl, respectively, required to dissociate the complex. On the other hand we could not detect the binding of thioredoxin to gene 2.5 protein, even in the absence of NaCl. These results suggest that the binding of thioredoxin to gene 5 protein alters the conformation of gene 5 protein, converting it to a structure more accessible for binding to gene 2.5 protein.

Gene 2.5 protein binds to single-stranded DNA and gene 5 protein with comparable affinities, the dissociation constant for each being \( \sim 1 \mu \text{M} \) (this study and Kim et al., 1992). On the other hand, gene 5 protein binds to thioredoxin stronger by a factor of 200, having a dissociation constant of 5 mM (Huber et al., 1986). The binding of the gene 5 protein-thioredoxin complex for single-stranded DNA ranges between 1 and 80 mM in the absence of a primer, and \( \sim 1 \mu \text{M} \) for a specific primer-template complex (Huber et al., 1987). Thus, the interactions between: 1) gene 2.5 protein and gene 5 protein-thioredoxin complex, 2) gene 2.5 protein and single-stranded DNA, and 3) gene 5 protein-thioredoxin and single-stranded DNA, are all of a similar strength, each weaker by a factor of 100-1,000 than the interactions between: 1) gene 5 protein and thioredoxin, and 2) gene 5 protein-thioredoxin and a primer-template.

For what purposes does gene 2.5 protein bind to T7 DNA polymerase? Three activities are attributed to the gene 2.5 protein. 1) It is a single-stranded DNA-binding protein that stimulates T7 DNA polymerase activity on single-stranded DNA (Reuben and Geffcr, 1973; Scherzinger et al., 1973; this study); 2) it stimulates the synthesis of RNA primers catalyzed by T7 primase, and increases the efficiency with which these primers are used by T7 DNA polymerase to initiate lagging strand synthesis (Nakai and Richardson, 1988); and 3) it is a component of the recombination mechanism in T7 (Araki and Ogawa, 1981a). Below, we discuss the possible relevance of a specific interaction between gene 2.5 protein and T7 DNA polymerase with respect to each of these roles.

T7 gene 5 protein is a DNA polymerase with very low processivity (Tabor et al., 1987). Thioredoxin binds to gene 5 protein and greatly increases its processivity on single-stranded DNA. However, the gene 5 protein-thioredoxin complex alone is unable to synthesize through double-stranded DNA. Single-stranded DNA such as M13 DNA has regions of secondary structure which impede T7 DNA polymerase in a manner analogous to that of double-stranded DNA. We demonstrate here that T7 gene 2.5 protein, E. coli SSB protein, and T4 gene 32 protein all increase the processivity of the gene 5 protein-thioredoxin complex on single-stranded DNA, albeit to different extents. This is consistent with the reported role of each of these proteins as a single-stranded DNA-binding protein that removes the secondary structure from single-stranded DNA. E. coli SSB protein is clearly the most effective of the three proteins tested at stimulating synthesis by T7 DNA polymerase and eliminating pause sites on a single-stranded DNA template. T7 gene 2.5 protein is
also effective, although significantly less so than E. coli SSB protein. T4 gene 32 protein is the least effective of the three proteins at stimulating the processivity of T7 DNA polymerase.

The differences observed in stimulation of T7 DNA polymerase by the different binding proteins cannot be explained by the relative binding affinities to single-stranded DNA since E. coli SSB protein and T4 gene 32 protein each bind to single-stranded DNA 25 times tighter than T7 gene 2.5 protein. Perhaps the stimulation of T7 DNA polymerase by E. coli SSB protein and T7 gene 2.5 protein is in part a result of specific interactions with T7 DNA polymerase since T7 phase has evolved in association with these two proteins, but not with T4 gene 32 protein. The interaction we describe here between T7 gene 2.5 protein and T7 DNA polymerase could facilitate the movement of the polymerase along single-stranded DNA during DNA synthesis. An interaction between T7 DNA polymerase and E. coli SSB protein based on sedimentation analysis has been reported (Molineux and Gettur, 1975). T4 DNA polymerase is stimulated specifically by T4 gene 32 protein (Huberman et al., 1971; Nossal and Peterlin, 1979), and a specific interaction between these two proteins has been shown both by sedimentation analysis (Huberman et al., 1971) and affinity chromatography (Formosa et al., 1983).

Historically, “single-stranded DNA-binding proteins” have referred to proteins whose role has been thought to bind to single-stranded DNA and remove secondary structures, thus facilitating the movement of DNA polymerases and other enzymes that interact with single-stranded DNA. Although E. coli recA protein binds to single-stranded DNA, it is not referred to as a single-stranded DNA-binding protein since its principle function is the catalysis of homologous pairing and strand uptake (see Radding, 1991). In the present study we measured the effect of E. coli recA protein on the stimulation of T7 DNA polymerase, an activity associated with single-stranded DNA-binding proteins. E. coli recA protein in fact inhibits DNA synthesis and the processivity of T7 DNA polymerase.

T7 gene 2.5 protein also has been shown to stimulate the synthesis of oligonucleotide primers by T7 primase and increase the efficiency with which these primers are used by T7 DNA polymerase to initiate lagging strand synthesis (Nakai and Richardson, 1988; Mendelman and Richardson, 1991). Specific interactions between gene 2.5 protein and both T7 primase and T7 DNA polymerase would be expected to facilitate both of these activities. In addition to the specific interactions demonstrated here between gene 2.5 protein and T7 DNA polymerase, we have shown by affinity chromatography that the gene 2.5 protein binds specifically to the T7 primase.

Finally, both T7 gene 2.5 protein (Araki and Ogawa, 1981a) and T7 DNA polymerase (Powlung and Knipper, 1974; Kerr and Sadowski, 1975) have been implicated in recombination in phage T7-infected cells. In phage T4 homologous base pairing is catalyzed by T4 uvsX protein, with the single-stranded DNA taken up into the duplex serving as a primer for T4 DNA polymerase (Formosa and Alberts, 1986a). This pathway serves two functions in T4; it is the primary pathway for recombination in T4, and it is a mechanism for the initiation of T4 DNA replication, particularly late in its life cycle (Mosig, 1983). T4 gene 32 protein, the single-stranded DNA-binding protein of T4, stimulates these activities (Yonesaki and Minagawa, 1985; Formosa and Alberts, 1986a, 1986b) and has been shown to bind specifically to both T4 uvsX protein and T4 DNA polymerase (Formosa et al., 1983). The specific interaction we have shown between T7 gene 2.5 protein and T7 DNA polymerase may play an important role in recombination in phage T7-infected cells.

Acknowledgments—We thank Lynn Mendelman and Susannah Wurgtler for critical reading of the manuscript.

REFERENCES