Protein-Protein and Protein-DNA Interactions at The Bacteriophage T4 DNA Replication Fork. Characterization of a Fluorescently Labeled DNA Polymerase Sliding Clamp

Daniel J. Sexton  
*Pennsylvania State University*

Theodore E. Carver  
*Pennsylvania State University*

Anthony J. Berdis  
*Cleveland State University, A.BERDIS@csuohio.edu*

Stephen J. Benkovic  
*Pennsylvania State University*

Follow this and additional works at: [https://engagedscholarship.csuohio.edu/scichem_facpub](https://engagedscholarship.csuohio.edu/scichem_facpub)

Part of the Biochemistry Commons, and the Chemistry Commons

*How does access to this work benefit you? Let us know!*

**Recommended Citation**
Sexton, Daniel J.; Carver, Theodore E.; Berdis, Anthony J.; and Benkovic, Stephen J., "Protein-Protein and Protein-DNA Interactions at The Bacteriophage T4 DNA Replication Fork. Characterization of a Fluorescently Labeled DNA Polymerase Sliding Clamp" (1996). *Chemistry Faculty Publications*. 206.  
[https://engagedscholarship.csuohio.edu/scichem_facpub/206](https://engagedscholarship.csuohio.edu/scichem_facpub/206)

This Article is brought to you for free and open access by the Chemistry Department at EngagedScholarship@CSU. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.
Protein-Protein and Protein-DNA Interactions at the Bacteriophage T4 DNA Replication Fork

CHARACTERIZATION OF A FLUORESCENTLY LABELED DNA POLYMERASE SLIDING CLAMP

(Received for publication, May 28, 1996, and in revised form, August 3, 1996)

Daniel J. Sexton, Theodore E. Carver, Anthony J. Berdis, and Stephen J. Benkovic‡
From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

The T4 DNA polymerase holoenzyme is composed of the polymerase enzyme complexed to the sliding clamp (the 45 protein), which is loaded onto DNA by an ATP-dependent clamp loader (the 44/62 complex). This paper describes a new method to directly investigate the mechanism of holoenzyme assembly using a fluorescently labeled cysteine mutant of the 45 protein. This protein possessed unaltered function yet produced substantial changes in probe fluorescence intensity upon interacting with other components of the holoenzyme. These fluorescence changes provide insight into the role of ATP hydrolysis in holoenzyme assembly. Using either ATP or the non-hydrolyzable ATP analog, adenosine 5'-O-(3-thiophosphate), events in holoenzyme assembly were assigned as either dependent or independent of ATP hydrolysis. A holoenzyme assembly mechanism is proposed in which the 44/62 complex mediates the association of the 45 protein with DNA in an ATP-dependent manner not requiring ATP hydrolysis. Upon ATP hydrolysis, the 44/62 complex triggers a conformational change in the 45 protein that may be attributed to the clamp loading onto DNA.

In most biological systems, the replication of DNA involves the coordinated actions of a multitude of different proteins. In general, as the complexity of the organism increases so do the number of proteins participating in DNA replication. The bacteriophage T4 DNA replication system is well suited for a mechanistic investigation of the protein-protein interactions required for replication due to the relatively low number of proteins involved and the apparent functional similarities to other more complicated systems such as those found in *Escherichia coli* and eucaryotes (reviewed in Refs. 1 and 2).

The bacteriophage T4 replication fork is comprised of the DNA polymerase enzyme (the product of gene 43), the DNA polymerase accessory proteins (the 44/62 complex and the 45 protein), as well as the ssDNA-binding protein (the 32 protein), the helicase (the 41 protein), the primase (the 61 protein), and the helicase accessory protein (the 59 protein) (3, 4). The T4 DNA polymerase holoenzyme consists of the T4 polymerase, the helicase (the 41 protein), the primase (the 61 protein), and the ssDNA-binding protein (the 32 protein), which possesses both a 5'-3' DNA polymerase holoenzyme consists of the T4 polymerase, the helicase accessory protein (the 59 protein) (3, 4). The T4 DNA polymerase holoenzyme consists of the T4 polymerase, the helicase (the 41 protein), the primase (the 61 protein), and the ssDNA-binding protein (the 32 protein), which possesses both a 5'–3' DNA polymerase holoenzyme. ATP hydrolysis, the 44/62 complex triggers a conformational change in the 45 protein that may be attributed to the clamp loading onto DNA.

© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available online at http://www-jbc.stanford.edu/jbc/

This work was supported by a National Institutes of Health Grant GM13306 (to S. J. B.). 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 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Chemistry, 152 Davey Laboratory, Pennsylvania State University, University Park, PA 16802. Tel.: 814-865-2882; Fax: 814-865-2973. E-mail: sjb1@psu.edu.

‡To whom correspondence should be addressed: J. Kuriyan, personal communication.

The abbreviations used are: ATPγS, adenosine 5′-O-(3-thiophosphate); bio, biotin; DCIA, 7-diethylamino-3-(4′-iodoacetyl)amino)phenyl)-4-methylcoumarin; DTNB, 5,5′-dithio-bis(2′,2′-nitrobenzoic acid); IAANS, 2-(4′-((iodoacetamido)unilino)naphthalene-6-sulfonic acid, sodium salt; IAEADANS, 5-(((iodoacetamido)ethyl)amino)naphthalene-1-sulfonic acid; IANBD, N-((2-(iodoacetoxyl)ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; IAF, 5-iodoacetamidofluorescein.

1 J. Kuriyan, personal communication.

2 The abbreviations used are: ATPγS, adenosine 5′-O-(3-thiophosphate); bio, biotin; DCIA, 7-diethylamino-3-(4′-iodoacetyl)amino)phenyl)-4-methylcoumarin; DTNB, 5,5′-dithio-bis(2′,2′-nitrobenzoic acid); IAANS, 2-(4′-((iodoacetamido)unilino)naphthalene-6-sulfonic acid, sodium salt; IAEADANS, 5-(((iodoacetamido)ethyl)amino)naphthalene-1-sulfonic acid; IANBD, N-((2-(iodoacetoxyl)ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; IAF, 5-iodoacetamidofluorescein.
possess a measurable intrinsic activity. It would therefore be advantageous to have a means of directly monitoring the 45 protein interactions during complex formation.

In this paper, we describe a fluorescently labeled cysteine mutant of the 45 protein. It is demonstrated that this fluorescently labeled 45 protein provides a novel means for the direct investigation of certain 45 protein-protein interactions as well as 45 protein-DNA interactions. The observed fluorescence changes enabled the presentation of a partial mechanism for T4 holoenzyme assembly. The change in the fluorescence of the labeled 45 protein that occurs upon holoenzyme formation has also provided a new assay for the determination of the T4 holoenzyme-DNA complex dissociation rate constant.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized with an Expedite 8909 DNA synthesizer (Perceptive Biosystems) and purified according to Capon et al. (18). Biotin-labeled oligonucleotides were prepared using a BioTEG phosphoramidite as obtained from Glen Research. The fluorescently labeled oligonucleotides (DEADANS, IANBD, IAF, IAANS, 2-(4-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium salt) were obtained from Molecular Probes, and pyrene maleimide was obtained from Acrors. ATP-S was purchased from Boehringer Mannheim as a 98% pure solution and used without further purification. DuPont NEN was the source of the [γ-32P]ATP used to 5' end label oligonucleotides. The T4 polymerase (U. S. Biochemical Corp.). All other biochemicals and chemicals were obtained from Sigma or Fisher and were of analytical grade or better.

DNA encoding a threonine to cysteine mutation at position 7 in the 45 protein (TCT-45 protein) ligated into a pET 26b expression vector was a generous gift of Dr. John Kuriyan (Rockefeller University). Following transformation of the DNA into BL21 DE3 E. coli and IPTG induction, the T7C-45 protein was purified according to Nosal (19). The wild type 45 protein and the 44/62 complex were prepared by overproducing strains obtained from Dr. William Konigsberg (Yale University) as described previously (19). Following purification, the T4 exonuclease-deficient polymerase D219A mutant was purified according to Frey et al. (20). The D219A mutant of the T4 polymerase was used in these studies to avoid complications arising from the presence of the exonuclease activity. The polymerase activity of the T4 D219A polymerase is identical to the wild type enzyme (20).

Primer Template Construction—The biotinylated 34/62/36-mer DNA substrate was constructed as described previously (12). This DNA substrate is composed of a 34-mer primer annealed to a 3-mer with a 36-mer fork strand annealed to the 5' end of the template with an 18-base overhang.

T7C-45 Protein Cysteine Modification—The modification of the cysteine thiol of the T7C-45 protein was performed upon passage of the protein solution over a G-25 column equilibrated with degassed buffer A (50 mM HEPES, 150 mM KOAc, 1 mM EDTA, 10% glycerol, pH 7.8) followed by the addition of a 10-fold molar excess of thiol reagent over protein monomer. The cysteine labeling reaction was allowed to proceed approximately 4 h at 4°C. Excess thiol reagent was removed by extensive dialysis against buffer B (50 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, pH 8.0). The labeled protein solution was then concentrated in a microconcentrator (Centricron-30) and stored at −80°C. The modification of cysteine residues was quantitated by measuring the dye concentration from its molar extinction at a wavelength other than 280 nm. Protein concentration was determined using the Bradford assay with the 45 protein as the standard. Labeling efficiencies are reported as mol of dye/mol of 45 trimer.

Steady State ATPase Assay—Steady state ATP hydrolysis measurements were performed using a phosphoendopyruvate kinase/actate dehydrogenase enzyme coupled system where the consumption of ATP was monitored spectrophotometrically (OLS-Cary-14 spectrophotometer) upon oxidation of NADH (13). ATPase activity was observed upon addition of the 45 protein (250 nm) to a solution containing 250 mM 44/62 complex, 250 mM biotinylated 34/62/36-mer DNA, 1 µM streptavidin, 1 mM ATP, 10 mM phosphoenolpyruvate, 200 mM NADH, 6 units of phosphoendopyruvate kinase/actate dehydrogenase mix, and biotinylated 34/62/36-mer DNA (150 mM potassium acetate, 10 mM magnesium acetate, 10 mM β-mercaptoethanol, and 25 mM Tris, pH 7.5) at 25°C. The T4 D219A polymerase protein was subsequently added to observe the inhibition of the ATP hydrolysis upon stable complex formation. The effect of the mutation of the threonine at position 7 to a cysteine in the 45 protein (the TCT-45 protein) as well as the subsequent thiol derivatization of the T7C thiol on protein function was assessed by substitution for wild type, unlabeled 45 protein. Initial rates of ATP hydrolysis were obtained under conditions where less than 10% of the limiting reactant was utilized over the reaction time course.

Pre-steady State ATPase Assay—The pre-steady state ATPase rates were performed using a rapid quench instrument (21) as described previously (13). The ATPase rates were obtained from an assay mixture containing 250 mM 44/62 complex, 250 mM 45 protein (or labeled 45 protein 34/62/36-mer biotinylated DNA, 50 nM ATP, 50 nM [γ-32P]ATP in buffer C at 25°C. Briefly, the reactions were quenched with 1 mM HCl and neutralized with an appropriate amount of 3 M NaOH in 1 mM Tris base followed by a thin layer chromatoographic separation of [γ-32P]ATP from γ-32P and then radiochemical analysis using a Molecular Dynamics Phosphorimager.

DNA Fluorescently Labeled DNA Polymerase Sliding Clamp—A strand displacement assay was utilized to further characterize the effect of T7C-45 protein thiol modification on the formation of active holoenzyme. This assay has been described in detail elsewhere (12). Briefly, an assay mixture containing 50 mM 32P-5'-end-labeled, biotinylated 34/62/36-mer DNA, 55 mM streptavidin, 1 mM ATP, and 10 µM dCTP is preincubated 30 s with 10 nM T4 D219A polymerase, 55 nM 44/62 complex, 55 nM 45 protein before addition of 10 µM remaining deoxynucleotides (dATP, dTTP, and dGTP) and 1 mg/ml salmon sperm single strand DNA trap. Following the addition of the remaining dNTPs and single strand DNA trap, aliquots were removed at different times and quenched in 2 mM HCl and extracted with phenol/chloroform/isooamyl alcohol (25:24:1). The zero point was obtained by omitting the protein from the assay mixture. The omission of the remaining three nucleotides was an experimental control to assess the incorporation extent of the first nucleotide. Prior to loading onto a 16% polyacrylamide, 8 x urea, sequencing gel, the samples were neutralized with the addition of an appropriate amount of 3 M NaOH in 1 mM Tris-base. The sequencing gels were exposed to constant electrophoresis, and the distribution of the radioactivity was analyzed using a Molecular Dynamics Phosphorimager.

Steady State Fluorescence—Steady state fluorescence measurements were performed using an SLM Amino 8000C photon counting spectrophotometer equipped with a thermostated cell compartment that was maintained at 25°C. The experiments were performed in buffer C in the presence of an appropriate amount of T7C-45-ANBD protein to provide a satisfactory fluorescence signal (usually 250 nM). The effects of the other components of the T4 polymerase holoenzyme (i.e. proteins, DNA, and/or ATP) on the T7C-45-ANBD fluorescence were observed upon their direct addition to the fluorescence cuvette. The fluorescence spectra obtained were normalized for the effects of dilution.

Stopped-flow Fluorescence—Stopped-flow fluorescence measurements were performed using an Applied Photophysics stopped-flow instrument at a constant temperature of 25°C. The changes in fluorescence were observed upon stopped-flow mixing of syringes A and syringe B. Syringe A contained 500 nM T7C-45-ANBD protein, 500 nM of the 44/62 complex, 1 mM ATP, 500 nM biotinylated 34/62/36-mer DNA, 50 nM T4 D219A polymerase, and 550 mM streptavidin in buffer C. Syringe B contained 20 mM glucose and 20 units/ml glucose hexokinase in buffer C. The concentration of protein and DNA in the two syringes of the stopped-flow instrument was diluted by a factor of 2 upon stopped flow mixing.

Data Analysis—Data obtained from the pre-steady state rate of ATP hydrolysis by the 44/62 complex were fit to Equation 1:

\[ y = A e^{-B t} + C \]

(1)

where \( A \) is the amplitude of the pre-steady state phase, \( B \) is the pre-steady state rate constant, and \( C \) is a constant. Data obtained from the stopped-flow fluorescence determination of the kinetics of holoenzyme dissociation were best fit by Equation 2:

\[ y = A e^{-B t} + Ct + D \]

(2)

where \( A \) is the amplitude of the exponential decay, \( B \) is the exponential decay rate constant, \( C \) is a linear decay rate, and \( D \) is a constant.

RESULTS

Fluorescent Labeling of the 45 Protein—The wild type 45 protein is devoid of cysteine residues. The site-selective introduction of a cysteine residue into the sequence of the 45 protein enables the covalent attachment of a thiol-reactive fluorescent molecule at a known position in the primary structure. A mutated 45 protein, in which a threonine residue at position 7...
Fluorescently Labeled DNA Polymerase Sliding Clamp

Table I

| Thiol reagent | Probe:trimer* |
|---------------|--------------|
| DCIA          | 0.6          |
| Dibromobimane | 2.3          |
| DTNB          | 3.0          |
| IAANS         | 0.4          |
| IAEDans       | 1.3          |
| IAF           | 1.5          |
| IANBD         | 0.9          |
| Pyrene maleimide | 1.2  |

*The labeling efficiency was determined as described under “Experimental Procedures” where the concentration of each probe was determined using the following molar extinction coefficients: DCIA, 33,000 liter · cm · mol⁻¹ at 382 nm; dibromobimane, 5,300 liter · cm · mol⁻¹ at 397 nm; DTNB, 13,600 liter · cm · mol⁻¹ at 412 nm; IAANS, 26,000 liter · cm · mol⁻¹ at 329 nm; IAEDans, 5,700 liter · cm · mol⁻¹ at 336 nm; IAF, 82,000 liter · cm · mol⁻¹ at 491 nm; IANBD, 23,000 liter · cm · mol⁻¹ at 472 nm and pyrene maleimide, 38,000 liter · cm · mol⁻¹ at 339 nm.

A rapid chemical quench method has been developed to measure the pre-steady state kinetics of ATP hydrolysis by the 44/62 complex (13). The ability of the T7C-45-ANBD protein to substitute for the wild type 45 protein under pre-steady state conditions was also assessed (Table II). No significant difference was observed between the T7C-45-ANBD protein and the wild type 45 protein with respect to the measured pre-steady state rates and the burst amplitudes (Table II). The pre-steady state ATPase rate is determined by the chemical rate of ATP hydrolysis rather than product release (steady state rate). The burst amplitude relative to the 44/62 complex concentration provides the stoichiometry of ATP consumption. At a concentration of 250 nM the burst amplitude for both forms of the 45 protein was approximately 1 μM which corresponds to 4 mol of ATP hydrolyzed per mol of 44/62 complex as previously reported (13).

To further examine the effect of the T7C mutation and subsequent thiol derivatization of the 45 protein on holoenzyme function, a strand displacement assay was utilized. In this assay the fork strand (36-mer) of a 5'-32P-biotinylated 62/34/36-mer is capable of being displaced by the holoenzyme but not by the T4 polymerase alone (11, 12). Displacement of the fork strand gives rise to the production of a fully extended 62-mer primer strand that can be differentiated from the bio62-mer template strand on a polyacrylamide sequencing gel. Quantitation of the radioactivity of the 62-mer primer strand yields a measure of the ability of the T4 replicative proteins to form productive holoenzyme complex. Fig. 1 demonstrates that the T7C-45 mutant protein displays strand displacement activity comparable with the wild type 45 protein even when derivatized with IANBD.

Steady State Fluorescence of T7C-45-ANBD—The T7C-45-ANBD protein was selected for further study based on two important properties. First, the ability of the T7C-45-ANBD protein to substitute for the wild type 45 protein in the assays described above, without a measurable loss of activity (Table II and Fig. 1), makes this labeled 45 protein potentially suitable for monitoring the interactions of the 45 protein at the T4 replication fork. Second, the environmentally sensitive nature of T7C-45-ANBD has been shown to permit the detection of certain 45 protein-44/62 complex and 45 protein-DNA interactions (Fig. 2). As shown in Fig. 2, the addition of the 44/62 complex to a solution of the T7C-45-ANBD protein resulted in no measurable change in the fluorescence emission spectrum. The subsequent addition of ATP to the above solution containing T7C-45-ANBD and 44/62 complex caused a marked (approximately 1.7-fold) increase in the fluorescence intensity. In the absence of the 44/62 complex ATP had no effect on the fluorescence of the T7C-45-ANBD protein (data not shown). That fluorescence increase was then attenuated by the addition of the bio34/62/36-mer to an intermediate level of fluorescent intensity approximately 1.3 times greater than that of the T7C-45-ANBD alone. The fluorescence of the T7C-45-ANBD protein is insensitive to the addition of the T4 polymerase whether it is added to the mixture of T7C-45-ANBD protein, 44/62 complex, and DNA (Fig. 2) or in any other order of addition (data not shown).

A demonstration of whether or not ATP hydrolysis is required for a particular biochemical event is often made possible by testing the ability of nonhydrolyzable ATP analogs to substitute for ATP. ATP-γS has been shown to be a potent inhibitor of the 44/62 complex ATPase activity, whereas the ATP analogs AMP-PNP and AMP-PCP are much weaker inhibitors (14).

3 The T7C-45 mutant protein was obtained from the Kuriyan Laboratory (Rockefeller University).

4 A. J. Berdis, unpublished data.
The ATP analogs, AMP-PNP and AMP-PCP, did not produce any change in the fluorescence of the T7C-45-ANBD protein under the above experimental conditions and at a concentration of up to 10 mM ATP analog (data not shown).

At a concentration of 1 mM, ATP$\gamma$S was not able to elicit all of the fluorescence changes mediated by ATP in Fig. 2. However, there is a fluorescence increase upon the addition of DNA to a mixture containing T7C-45-ANBD and the 44/62 complex in the presence of ATP$\gamma$S (Fig. 3). That fluorescence increase is not affected by the presence or absence of T4 polymerase or the order of addition (data not shown). Magnesium ion (10 mM) was required to observe the ATP$\gamma$S-dependent fluorescence change.

**The Stoichiometry of the T7C-45-ANBD:44/62 Interaction**—The stoichiometry of the ATP-dependent interaction between the T7C-45-ANBD protein and the 44/62 complex was determined by varying the concentration of 44/62 complex. As shown in Fig. 4, the increase in fluorescence reaches a plateau at approximately a 1:1 molar ratio of 44/62 complex to T7C-45-ANBD protein.

The Stoichiometry of the T7C-45-ANBD:44/62 Interaction—The stoichiometry of the ATP-dependent interaction between the T7C-45-ANBD protein and the 44/62 complex was determined by varying the concentration of 44/62 complex. As shown in Fig. 4, the increase in fluorescence reaches a plateau at approximately a 1:1 molar ratio of 44/62 complex to T7C-45-ANBD protein.

**Holoenzyme-DNA Dissociation Rate Constant**—Stopped-flow fluorescence rapid mixing experiments were performed to measure the dissociation rate of the holoenzyme from the bio34/62/36-mer. In order to measure the holoenzyme dissociation rate, the holoenzyme was assembled onto the DNA substrate in one syringe and pushed against another syringe containing excess glucose and hexokinase that rapidly consume the ATP. Under the experimental conditions (10 units/ml hexokinase) the ATP (1 mM) should be consumed in approximately 6 s. The approximate dead time of the experiment is therefore 6 s. The data obtained are shown in Fig. 5 where it can be seen that after stopped-flow mixing of the assembled holoenzyme-DNA complex with glucose and hexokinase there is a decrease in fluorescence that can be approximated by a single exponential decay (0.011 ± 0.002 s$^{-1}$) followed by a linear decrease ($7.8 \times 10^{-5} + 2 \times 10^{-6}$ g$^{-1}$). The single exponential decay rate represents the dissociation rate constant of the holoenzyme-

---

**Table II**

| 45 protein | Steady state rate a | Steady state rate b | Pre-steady state rate c | Pre-steady state amplitude d |
|------------|---------------------|---------------------|-------------------------|-----------------------------|
| WT 45      | 398                 | 87                  | 3.1                     | 0.9                         |
| T7C-45     | 421                 | 110                 | ND                      | ND                          |
| T7C-ANBD   | 428                 | 115                 | 3.9                     | 1.2                         |
| T7C-AF     | 228                 | 103                 | ND                      | ND                          |

---

*a* Steady state ATPase activity was performed as described under “Experimental Procedures.” Steady state rate 1 corresponds to the ATPase rate resulting from the stimulation by the 45 protein, and steady state rate 2 is the rate observed upon addition of the D219A T4 polymerase to the solution that contains the 45 protein. The difference between these values and those reported in Berdis and Benkovic (13) may be due to different preparations of the same DNA substrate.

*b* Pre-steady state ATPase measurements were performed as described under “Experimental Procedures.”

*c* ND, not determined.

---

**FIG. 1.** Strand displacement assay in the presence of the T7C-45-ANBD protein. The above sequencing gel (16% acrylamide, 8 M urea) was used to separate the polymerase extension products using the $^{32}$P-5'-end-labeled, biotinylated 34/62/36-mer DNA substrate. The lanes in A are the extension products observed in the presence of the polymerase alone (i.e. no accessory proteins), at different time points (0, 10, 20, and 30 s) plus a control point. The control point represents the incorporation of just the first nucleotide. B represents the results of the holoenzyme with wild type 45 protein, and C and D represent the observed results of the holoenzyme when the T7C-45 protein and the T7C-45-ANBD protein, respectively, were substituted for the wild type 45 protein. Strand displacement was observed according to the appearance of the fully extended primer as a 62-mer (indicated by the arrow) that was resolved from the template biotinylated 62-mer. The strand displacement experiment was performed as described under “Experimental Procedures.”

**FIG. 2.** Steady state fluorescence of the T7C-45-ANBD protein. The T7C-45-ANBD steady state fluorescence was observed in buffer C with 275 nM streptavidin at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. Spectrum 1 was obtained in the presence of 250 nM T7C-45-ANBD protein, and spectrum 2 is the result of the addition of 250 nM of the 44/62 complex. Spectrum 3 contains 250 nM T7C-45-ANBD protein, 250 nM of the 44/62 complex, and 1 mM ATP. Spectrum 4 contains 250 nM T7C-45-ANBD protein, 250 nM of the 44/62 complex, 1 mM ATP, and 250 nM biotinylated 34/62/36-mer DNA, and spectrum 5 was recorded with 250 nM T7C-45-ANBD protein, 250 nM of the 44/62 complex, 1 mM ATP, 250 nM biotinylated 34/62/36-mer DNA, and 250 nM T4 D219A polymerase.
DISCUSSION

In this paper, we have succeeded in labeling a cysteine mutant of the sliding clamp (the 45 protein) with an environmentally sensitive, thiol-reactive fluorescent probe. This fluorescently labeled 45 protein was then demonstrated to be useful in monitoring certain 45 protein-protein and 45 protein-DNA interactions associated with the T4 replication fork.

Fluorescent Labeling of the 45 Protein—Since the wild type 45 protein is devoid of cysteine residues, it was expected that the introduction of a single cysteine residue by site-directed mutagenesis would result in the incorporation of a single thiol-reactive fluorescent probe per monomer. However, the extent of T7C 45 protein thiol modification was consistently less than that expected for a 45 protein trimer with one cysteine residue per monomer (Table I). The probe to protein trimer ratio for many of the thiol-reactive probes was approximately 1:1 which may indicate that the 45 protein occupies an asymmetrical conformation.

Asymmetry may be introduced in the 45 protein by the interconversion between open and closed conformations (Fig. 6). In this model, the reactivity of the T7C thiol is influenced by whether the 45 protein is in the putative open or closed conformation. The modification of the first cysteine thiol may weaken subunit interactions at one interface so that interconversion between the open and closed conformation favors subunit opening at that weakened interface. Assuming that the cysteine thiols of the T7C-45 protein are derivatized only when they are near an open subunit interface, the remaining two cysteines would remain unmodified. This hypothesis suggests that thiol derivatization alters the structure of 45 protein. It was therefore necessary to thoroughly determine the effects of thiol modification on the 45 protein function.

It should be noted that the existence of a mixed population of multiply labeled and unlabeled 45 proteins that combine to yield an average probe to protein trimer ratio of 1:1 cannot be completely disregarded at this time. However, under conditions of up to 50-fold molar excess of probe to protein thiol and at prolonged reaction times (24 h) additional equivalent thiols in the T7C-45 protein should be completely reacted. Moreover, the apparent similarity with respect to the extent of T7C-45 protein thiol modification with several of the different thiol reagents listed in Table I suggests that the observed ratio of

DNA complex, while the linear rate can be explained by a small amount of protein precipitation in the absence of ATP.

FIG. 3. Effect of ATPγS on the changes in the steady state fluorescence of the T7C-45-ANBD protein. The T7C-45-ANBD steady state fluorescence was observed in buffer C with 275 nm streptavidin at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. Spectrum 1 was obtained in the presence of 250 nm T7C-45-ANBD protein, and spectrum 2 is the result of the addition of 250 nm of the 44/62 complex. Spectrum 3 contains 250 nm T7C-45-ANBD protein, 250 nm of the 44/62 complex, and 1 mM ATPγS. Spectrum 4 contains 250 nm T7C-45-ANBD protein, 250 nm of the 44/62 complex, 1 mM ATPγS, and 250 nm D219A T4 polymerase, and spectrum 5 was recorded with 250 nm T7C-45-ANBD protein, 250 nm of the 44/62 complex, 1 mM ATPγS, 250 nm T4 polymerase, and biotinylated 34/62/36-mer DNA.

FIG. 4. The stoichiometry of the T7C-45-ANBD protein:44/62 complex interaction. The T7C-45-ANBD steady state fluorescence was observed in buffer C at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. The fluorescence intensity of 500 nm T7C-45-ANBD was monitored as a function of the 44/62 complex concentration in the absence of DNA. Fluorescence measurements were recorded immediately following addition of the 44/62 complex that ensured no significant ATP depletion.

FIG. 5. Determination of the T4 polymerase holoenzyme-DNA dissociation rate constant. Stopped-flow fluorescence was used to measure the T4 polymerase holoenzyme-DNA dissociation rate constant where syringe A contained 500 nm T7C-45-ANBD protein, 500 nm of the 44/62 complex, 1 mM ATP, 500 nm biotinylated 34/62/36-mer DNA, 500 nm T4 D219A polymerase, and 550 nm streptavidin in buffer C. Syringe B contained 20 mM glucose and 20 units/ml glucose hexokinase in buffer C. Stopped-flow mixing of syringe A and B resulted in the rapid depletion of ATP as well the dilution of the above concentrations by a factor of 2. The thick line represents a fit of the data to a single exponential followed by a steady state as described under “Experimental Procedures.”
There were two fluorescence changes induced in the T7C-45-ANBD protein by the addition of the 44/62 complex and ATP. One change was DNA-independent and the other was DNA-dependent. The DNA-independent change was triggered by the addition of the 44/62 complex and ATP and had an apparent stoichiometry of 1:1. The 1:1 stoichiometry of the T7C-45-ANBD complex indicates that the observed increase in fluorescence is likely due to complex formation rather than a catalytic event such as the 44/62 complex-catalyzed opening or closing of the 45 protein trimeric ring.

The ATP dependence and the inability of the nonhydrolyzable ATP analogs AMP-PNP, AMP-PCP, or ATPγS to substitute in the formation of this DNA-independent fluorescence change suggests two possible scenarios. One possibility is that the fluorescence change is the result of a conformational change induced by binding ATP that cannot be induced by the ATP analogs. A more likely explanation is that this conformational change is triggered by ATP. This apparent requirement for ATP hydrolysis suggests that the T7C-45-ANBD complex has undergone an ATP hydrolysis-driven conformational change, such as the opening of the 45 protein.

Currently, the identity of this ATP hydrolysis-dependent conformational change in the 45 protein has not been assigned.

**T7C-45-ANBD Protein-DNA Interactions**—The DNA-dependent change in T7C-45-ANBD fluorescence in the presence of ATP was attributable to the 44/62 complex catalyzed loading of the T7C-45-ANBD protein onto DNA. The assignment of this DNA-dependent fluorescence change was made by measuring its rate of dissociation upon ATP consumption. The observed rate of fluorescence decay (0.011 s⁻¹) was similar to that obtained for the holoenzyme-DNA dissociation constant using a strand displacement assay method (0.002 s⁻¹, 11). The difference in the dissociation rate constants may be attributed to the fact that the substantial decrease in the fluorescence signal was best approximated by a single exponential decay, whereas the data obtained from the strand displacement assay were better approximated by a double exponential decay where, in addition to the 0.002 s⁻¹ rate, there was a population (30%) that decayed at a faster rate (0.028 s⁻¹, 11). The weighted average of these rates (0.0097 s⁻¹) is very similar to that obtained here (0.011 s⁻¹) which suggests that the fluorescence assay is not sensitive to the presence of the two distinct holoenzyme species observable by the strand displacement method.

Interestingly, the DNA-dependent change in T7C-45-ANBD fluorescence was also induced when ATPγS was substituted for ATP. Other ATP analogs that were examined (AMP-PNP and AMP-PCP) did not induce this fluorescence change. In this case, ATPγS appears to provide a clue toward determining the role of ATP hydrolysis in holoenzyme assembly. This effect of ATPγS may suggest that ATP binding rather than hydrolysis is required for the 44/62 complex to chaperone the 45 protein onto DNA. This may also suggest that the T7C-45-ANBD fluorescence change is independent of whether the 45 protein is correctly loaded onto DNA (i.e. with ATP) or merely associated with DNA in some manner. It was previously shown by DNA footprinting (15) and protein-DNA cross-linking (16) that the 45 protein is complexed with DNA in the presence of the 44/62 complex and ATPγS. Since ATPγS is not able to support functional holoenzyme assembly (14), the 45 protein is likely incompletely loaded onto DNA in its presence.

**Partial Mechanism for Holoenzyme Assembly**—Based on the ATP hydrolysis dependence of the observed fluorescence changes in the presence and absence of DNA, a putative partial mechanism is proposed (Fig. 7). The proposed mechanism is incomplete due to the fact that the loading of the polymerase
more stable ring closed form on DNA concomitant with 44/62 complex release.

Comparison with the DNA Polymerases of Other Systems—The E. coli replication system sliding clamp (the β clamp) is loaded by a five protein complex termed either the DNAX complex or the γ complex. The five proteins that assemble to form the E. coli clamp loader are the δ, the δ′, the χ, the ψ, and either the γ or the τ subunit. DNAX complexes formed with either the γ or the τ subunit differ in their ability to hydrolyze ATP, underscoring the need for a conformational change that exposes the 62 protein for interaction with the 45 protein. ATP hydrolysis within the 45–44/62 complex causes a conformational change in the 45 protein that results in the functionally loaded 45 protein on DNA.

Acknowledgments—We are grateful to Ismail Moarefi and John Kuriyan for providing us with the cysteinyl mutant of the 45 protein.

REFERENCES

1. Kornberg, A., and Baker, T. (1992) DNA Replication, 2nd Ed., pp. 113–217, W. H. Freeman and Co., New York
2. Stillman, B. (1994) Cell 78, 725–728
3. Young, M. C., Reddy, M. K., and von Hippel, P. H. (1995) Biochemistry 34, 8675–8690
4. Nossal, N. G. (1992) PASEB J. 6, 871–878
5. Kong, X.-P., O'Nurst, R., O'Donnell, M., and Kuriyan, J. (1992) Cell 69, 425–437
6. Krishna, T. S. R., Kong, X.-P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Cell 79, 1233–1243
7. O'Donnell, M., O'Nurst, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) Nucleic Acids Res. 21, 1–3
8. O'Nurst, R., Stukenberg, P. T., and O'Donnell, M. (1991) J. Biol. Chem. 266, 21681–21686
9. Yoder, B. L., and Burgers, P. M. J. (1991) J. Biol. Chem. 266, 22689–22697
10. Rush, J., Lin, T.-C., Quinones, M., Spicer, E. K., Douglas, I., Williams, K. R., and Konigsberg, W. H. (1989) J. Biol. Chem. 264, 10943–10953
11. Kaboord, B. F., and Benkovic, S. J. (1995) Curr. Biol. 5, 149–157
12. Kaboord, B. F., and Benkovic, S. J. (1996) Biochemistry 35, 1084–1092
13. Berdis, A. J., and Benkovic, S. J. (1996) Biochemistry 35, 9253–9265
14. Piperno, J. R., and Alberts, B. M. (1978) J. Biol. Chem. 253, 5174–5179
15. Munn, M. M., and Alberts, B. M. (1991) J. Biol. Chem. 266, 20024–20033
16. Capson, T. L., Benkovic, S. J., and Nossal, N. G. (1991) Cell 65, 249–258
17. Gogel, E. F., Young, M. C., Kubasek, W. L., Jarvis, T. C., and von Hippel, P. H. (1992) J. Mol. Biol. 224, 395–412
18. Capson, T. L., Peliska, J. A., Kaboord, B. F., Frey, M. W., Lively, C., Dahlberg, M., and Benkovic, S. J. (1992) Biochemistry 31, 10943–10994
19. Nossal, N. G. (1979) J. Biol. Chem. 254, 6026–6031
20. Frey, M. W., Nossal, N. G., Capson, T. L., and Benkovic, S. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2579–2583
21. Johnson, K. A. (1986) Methods Enzymol. 146, 687–705
22. Ellman, G. L. (1958) Arch. Biochem. Biophys. 82, 70–77
23. Dallmann, H. G., Thimmig, R. L., and McHenry, C. S. (1995) J. Biol. Chem. 270, 29555–29562
24. Naktinis, V., O’Nurst, R., Wang, L., and O'Donnell, M. (1995) J. Biol. Chem. 270, 13358–13365
25. Xiao, H., Naktinis, V., and O'Donnell, M. (1995) J. Biol. Chem. 270, 13378–13383
Protein-Protein and Protein-DNA Interactions at the Bacteriophage T4 DNA Replication Fork: CHARACTERIZATION OF A FLUORESCENTLY LABELED DNA POLYMERASE SLIDING CLAMP
Daniel J. Sexton, Theodore E. Carver, Anthony J. Berdis and Stephen J. Benkovic

*J. Biol. Chem.* 1996, 271:28045-28051.
doi: 10.1074/jbc.271.45.28045

Access the most updated version of this article at [http://www.jbc.org/content/271/45/28045](http://www.jbc.org/content/271/45/28045)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/271/45/28045.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 24 references, 12 of which can be accessed free at [http://www.jbc.org/content/271/45/28045.full.html#ref-list-1](http://www.jbc.org/content/271/45/28045.full.html#ref-list-1)