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Protein-Protein and Protein-DNA Interactions at the Bacteriophage T4 DNA Replication Fork

CHARACTERIZATION OF A FLUORESCENTLY LABELED DNA POLYMERASE SLIDING CLAMP*

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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, which possesses both a 5′–3′ polymerase and a 3′–5′ exonuclease activity, and the 45 protein. The homotrimeric 45 protein is the processivity factor for the polymerase, and its x-ray structure is now known to resemble the "sliding clamp" ring structure previously shown for the analogous proteins from *E. coli* (β-clamp) and eucaryotes (proliferating cell nuclear antigen)[1] (5, 6).[1] The 45 protein is loaded onto DNA at a primer template junction through the ATP-dependent activity of the 44/62 protein complex. The 44/62 complex has a functional and a limited sequence homology (7) to the γ complex clamp loader of *E. coli* (8) and the RF-C complex of eucaryotes (9). The oligomeric stoichiometry of the 44/62 complex is four 44 subunits to one 62 subunit with the ATPase activity residing on each of the 44 subunits (10). Recently, it was quantitatively demonstrated that the 44/62 complex loads the 45 protein in a catalytic manner and also functions as a chaperone protein to ensure productive holoenzyme complex (11, 12). The amount of ATP consumed in the 44/62-dependent loading of the 45 protein onto primed DNA has recently been shown to be consistent with the hydrolyis of ATP at all four ATPase sites in the 44/62 complex (13).

Despite the wealth of information regarding the roles of the different T4 replication proteins in DNA synthesis, there remain a number of individual events or "microprocesses" for which mechanisms are undetermined. For example, by dividing the T4 replication activity into three processes, 1) the assembly of the holoenzyme, 2) the synthesis of DNA, and 3) the disassembly of the holoenzyme, it is evident that there exist events within each process that may be considered microprocesses. One such microprocess within the assembly of the holoenzyme concerns the development of the protein-protein interaction(s) between the 45 protein and the 44/62 complex that are required to load the 45 protein onto DNA. The mechanism by which the 44/62 complex loads the 45 protein onto DNA is not known. It is known that ATP hydrolysis is required for the stimulation of the T4 polymerize processivity by the accessory proteins (14). However, ATP hydrolysis appears not to be necessary for a 44/62 complex-mediated 45 protein-DNA interaction since this interaction was observed with the non-hydrolyzable ATP analog, ATPγS,[2] by both DNA footprinting (15) and protein-DNA cross-linking experiments (16). ATP hydrolysis was required to observe, by cyroelectron microscopy, structures (termed "hash marks") on nicked DNA that corresponded to 45 protein trimers (17) that emphasizes the importance of ATP hydrolysis in loading the 45 protein onto DNA. The investigation of the DNA loading mechanism of the 45 protein is made difficult by the fact that 45 protein does not

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[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)anilino)naphthalene-6-sulfonic acid, sodium salt; IAEDANS, 5-((((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; IANBD, *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-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 Capson *et al*. (18). Biotin-labeled oligonucleotides were prepared using a BioTEG phosphoramidite as obtained from Glen Research. The fluorescent probes DCIA, dibromobimane, IAEDANS, IANBD, IAF, IAANS, 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium salt were obtained from Molecular Probes, and pyrene maleimide was obtained from Acros. ATPγS was purchased from Boehringer Mannheim as a 98% pure solution and used without further purification. DuPont NEN was the source of the [γ-^{32}P]ATP used to 5′-end label oligonucleotides with T4 polynucleotide kinase (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 (T7C-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-induced expression, the T7C-45 protein was purified according to Nossal (19). The wild type 45 protein and the 44/62 complex were purified from overproducing strains obtained from Dr. William Konigsberg (Yale University) as described previously (19). 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 that of 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′-biotinylated 62-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 (Centricon-30) and stored at −80 °C. The extent of cysteine modification 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 phosphoenolpyruvate kinase/lactate dehydrogenase enzyme coupled system where the consumption of ATP was monitored spectrophotometrically (OLIS-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 nM 44/62 complex, 250 nM biotinylated 34/62/36-mer DNA, 1 μM streptavidin, 1 mM ATP, 10 mM phosphoenolpyruvate, 200 μM NADH, 6 units of phosphoenolpyruvate kinase/lactate dehydrogenase mix, and buffer C (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 T7C-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 nM 44/62 complex, 250 nM 45 protein (or labeled 45 protein), 500 nM biotinylated 34/62/36-mer DNA, 1 μM streptavidin, 1 mM ATP, 50 nM [γ-^{32}P]ATP in buffer C at 25 °C. Briefly, the reactions were quenched with 1 M HCl and neutralized with an appropriate amount of 3 M NaOH in 1 M Tris base followed by a thin layer chromatographic separation of [γ-^{32}P]ATP from γ-^{32}P and then radiochemical analysis using a Molecular Dynamics Phosphorimager.

Strand Displacement Assay—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 nM ^{32}P-5′-end-labeled, biotinylated 34/62/36-mer DNA, 55 nM 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 M HCl and extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The zero point was obtained by omitting the protein from the assay mixture. The omission of the remaining three nucleotides provided an experimental control to assess the incorporation extent of the first nucleotide. Prior to loading onto a 16% polyacrylamide, 8 M urea, sequencing gel, the samples were neutralized with the addition of an appropriate amount of 3 M NaOH in 1 M Tris-base. The sequencing gels were exposed to constant current 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 Aminco 8000C photon counting spectrofluorometer 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 syringe 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, 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. 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:

$$y = Ae^{-Bt} + C \quad \text{(Eq. 1)}$$

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:

$$y = Ae^{-Bt} + Ct + D \quad \text{(Eq. 2)}$$

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

TABLE I
Thiol reagent labeling efficiency of the T7C-45 protein

Thiol reagent	Probe:trimer[a]
	mol/mol
DCIA	0.6
Dibromobimane	2.3
DTNB	3
IAANS	0.4
IAEDANS	1.3
IAF	1.5
IANBD	0.9
Pyrene maleimide	1.2

[a] 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 pyrenemaleimide, 36,000 liter · cm · mol^{-1} at 339 nm.

from the N terminus, was site-specifically mutated to a cysteine residue producing the threonine 7 to cysteine 45 mutant protein (T7C-45 protein). The details for the creation of this cysteine mutant will be published elsewhere.[3]

The cysteine mutant, T7C-45 protein, was modified using several different thiol-reactive probes (Table I). The thiol quantitating reagent 5,5′-dithiobis(2,2′-nitrobenzoic acid) (DTNB; Ref. 22) reacted slowly with three of the thiols in the trimer indicating the presence of only the reduced form of the cysteines. No evidence of nonequivalent cysteine thiols in the T7C-45 protein trimer was provided by the DTNB reactivity since the reaction was best approximated by a single exponential (data not shown). However, thiol modification of the cysteine T7C-45 protein with the majority of the fluorescent probes consistently led to a probe:trimer ratio less than 3:1, the ratio expected if all thiols in the trimer were modified (Table I). It is evident that for most of the probes tested in this study the probe:trimer ratio was close to 1:1 (omitting DTNB, the average probe:trimer ratio equals 1.2:1) which suggests that only one out of three of the cysteines in the trimer is modified. Of the fluorescent probes tested in Table I, dibromobimane incorporated to the greatest extent (2.3:1) while the naphthalene derivative (IAANS) was the least reactive (0.4:1). A titration of the remaining thiols of the T7C-45-ANBD protein with DTNB yielded 2 thiols per trimer, which was again consistent with a labeling efficiency for IANBD of only 1 probe:trimer.

Activity of Fluorescently Labeled T7C-45 Protein—The measurement of the degree of stimulation of the 44/62 complex steady state ATPase activity by the fluorescently labeled T7C-45 proteins provides a convenient assay to assess the effects of derivatization on 45 protein function (Table II). ATP is consumed as the 44/62 complex loads the 45 protein onto the biotinylated 34/62/36 DNA substrate. In the absence of the T4 polymerase, the 45 protein dissociates from DNA allowing it to be reloaded by the 44/62 complex at the expense of additional ATP hydrolysis. The addition of the T4 polymerase diminishes ATP consumption through the formation of holoenzyme, which is more stable on DNA than the 45 protein alone (13). As shown in Table II, the T7C-45 mutant displays essentially identical activity to that of the wild type 45 protein. Labeling the T7C-45 protein with iodoacetamidofluorescein (IAF) led to a decrease in the observed stimulation of the ATPase activity of the 44/62 complex (Table II). The modification of the T7C-45 protein with IANBD, however, did not affect its ability to stimulate the 44/62 complex steady state ATPase activity (Table II).

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′-^{32}P-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. ATPγS 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).[4]

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

[4] A. J. Berdis, unpublished data.

TABLE II
Stimulation of the DNA-dependent 44/62 complex ATPase activity by fluorescently labeled 45 proteins

45 protein	Steady state rate 1[a]	Steady state rate 2[a]	Pre-steady state rate[b]	Pre-steady state amplitude[b]
	nM/s	*nM/s*	s^{-1}	*μM*
WT 45	398	87	3.1	0.9
T7C-45	421	110	ND[c]	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.

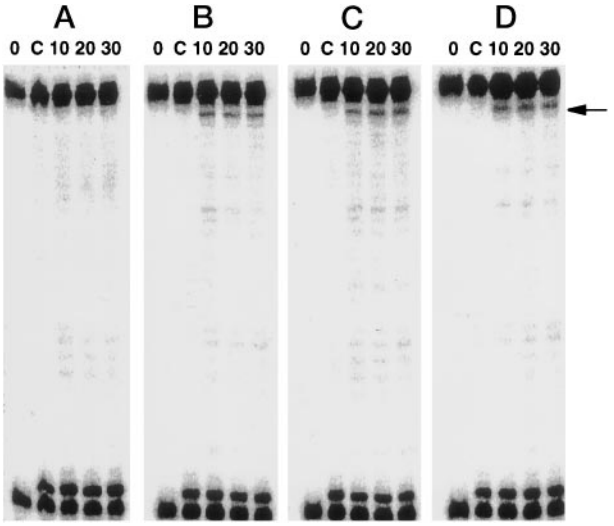

FIG. 1. **Strand displacement assay in the presence of the T7C-45-ANBD protein.** The above sequencing gel (16% acrylamide, 8 M urea) was used to separate the polymerase extension products using the ^{32}P-5′-end-labeled, biotinylated 34/62/36-mer DNA substrate. The lanes in *A* are the extension products 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."

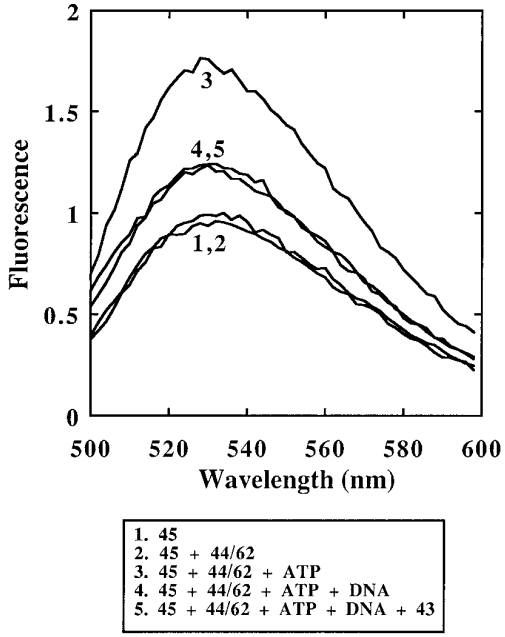

FIG. 2. **Steady state fluorescence of the T7C-45-ANBD protein.** The T7C-45-ANBD steady state fluorescence was observed in buffer C with 275 nM streptavidin at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. *Spectrum 1* was obtained in the presence of 250 nM T7C-45-ANBD 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.

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, ATPγS 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 ATPγS (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 ATPγS-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.

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 ($7.8 \times 10^{-5} \pm 2 \times 10^{-6}$ s^{-1}). The single exponential decay rate represents the dissociation rate constant of the holoenzyme-

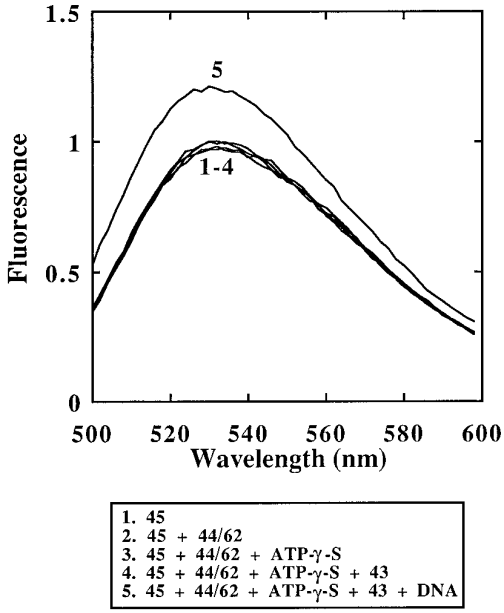

FIG. 3. **Effect of ATPγS on the changes in the steady state fluorescence of the T7C-45-ANBD protein.** The T7C-45-ANBD steady state fluorescence was observed in buffer C with 275 nM streptavidin at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. *Spectrum 1* was obtained 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.

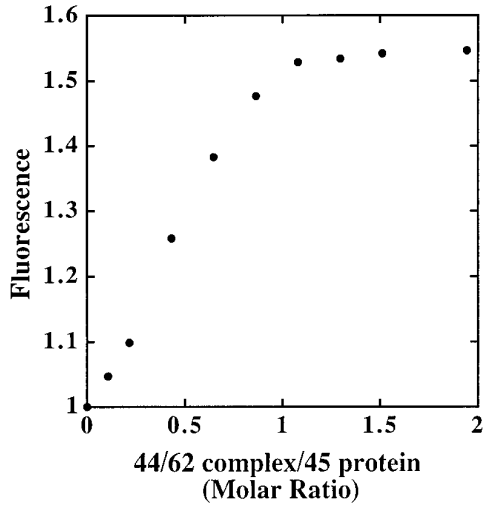

FIG. 4. **The stoichiometry of the T7C-45-ANBD protein:44/62 complex interaction.** The T7C-45-ANBD steady state fluorescence was observed in buffer C at 25 °C at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. The fluorescence intensity of 500 nM T7C-45-ANBD was monitored as 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.

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

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.

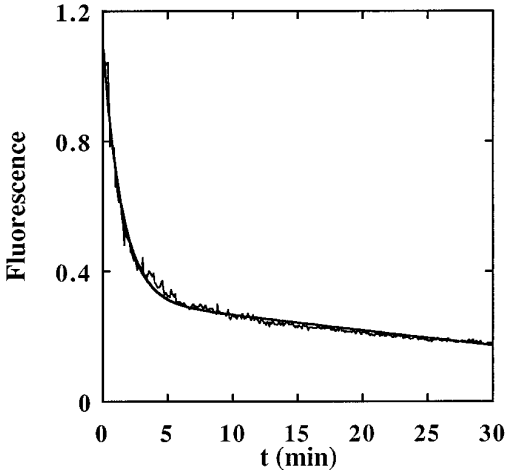

FIG. 5. **Determination of the T4 polymerase holoenzyme-DNA dissociation rate constant.** Stopped-flow fluorescence was used to measure the T4 polymerase holoenzyme-DNA dissociation rate constant where syringe A contained 500 nM T7C-45-ANBD protein, 500 nM of the 44/62 complex, 1 mM ATP, 500 nM biotinylated 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."

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

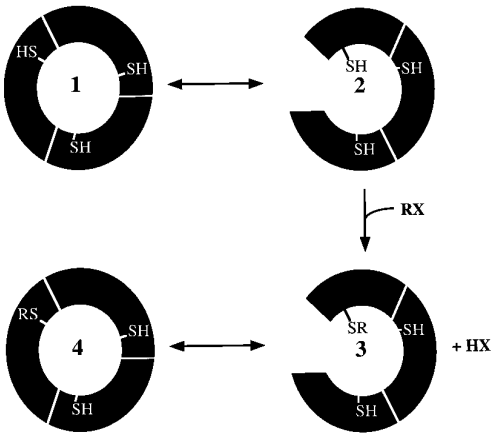

FIG. 6. **Model for the observed stoichiometry of T7C-45 protein thiol modification.** *Structure 1* represents the 45 protein in the closed conformation that may be in equilibrium with an open conformation (*structure 2*). *Structure 1* is shown with all cysteine thiols solvent inaccessible. Upon opening of the 45 protein at a single subunit interface, the environment of one cysteine thiol may change making one cysteine thiol more reactive. The reaction of this thiol with a thiol reagent (*RX*) may produce a 45 protein (*structures 3* and *4*) that is preferentially "opened" at the interface closest to the modified cysteine thiol.

probe:trimer is not due to the thiol reagent structure but rather some aspect of the protein itself. The ability of DTNB to modify all three cysteine thiols with equivalent kinetics may be attributed to its relatively small size.

Activity of Fluorescently Labeled T7C-45 Protein—The 45 protein does not possess a measurable catalytic activity by itself. Instead its function is assessed through either its stimulation of the DNA-dependent 44/62 ATPase activity or its ability to enhance the processivity of the T4 DNA polymerase. A steady state DNA-dependent ATPase assay provided a convenient assay for screening the effects of thiol modification. The T4 polymerase-45 protein interaction can be observed by the reduction in the ATP hydrolysis rate upon holoenzyme assembly. These measurements demonstrate that the 45 protein cannot tolerate large thiol reagents, such as the fluorescein derivative, at the T7C position, but it can tolerate small thiol reagents such as IANBD. This observation is supported by the x-ray crystal structure of the 45 protein that demonstrates that the T7 position is buried on the interior surface of the protein ring.[1] Interestingly, T7C-45 protein modification with the relatively large fluorescein derivative did not affect the T4 polymerase-induced ATPase shut down rate. This lack of an apparent effect on the holoenzyme ATPase rate by some large thiol reagents suggests that the T4 polymerase interacts at a site removed from the T7C position in the 45 protein.

Proper functioning of the T7C-45 protein labeled with IANBD (T7C-45-ANBD) was also demonstrated by a pre-steady state kinetic analysis of the stimulation of the DNA-dependent ATPase activity of the 44/62 complex and by a strand displacement assay. The pre-steady state ATPase measurements provided the ATPase burst rate and burst amplitude. It appears that it is the ATP hydrolysis burst rate that limits holoenzyme assembly (13, 11). The strand displacement assay demonstrated that the T7C-45 protein retains another function of the wild type 45 protein, namely the polymerase processivity enhancement.

T7C-45-ANBD Protein-Protein Interactions—The evaluation of several thiol-directed fluorescent probes led to the discovery that the T7C-45 protein modified with the environmentally sensitive probe, IANBD, produced substantial changes in fluorescence intensity upon interactions with the other components of T4 replication.

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·44/62 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·44/62 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

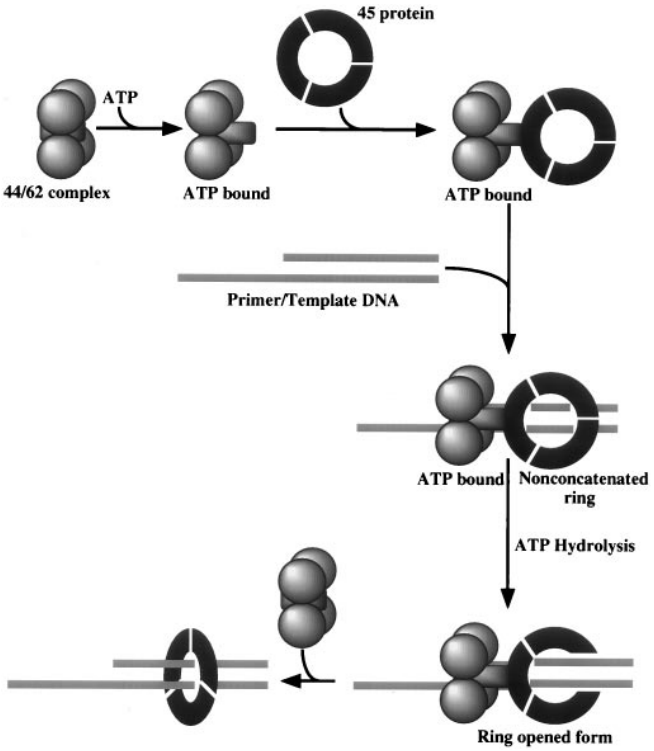

FIG. 7. **Partial mechanism for holoenzyme assembly.** The 44/62 complex is shown by four spheres that represent the 44 protein and one rectangle (the 62 protein). Upon binding ATP a conformational change is induced in the 44/62 complex that enables interaction with the 45 protein (trimeric ring). This 45 protein:44/62:ATP bound complex interacts with DNA without ATP hydrolysis to yield a noncatenated form of the 45 protein on DNA. The model has ring opening on DNA coupled to ATP hydrolysis. The opened form of the ring then decays to the closed ring on DNA with the release of the 44/62 complex.

does not cause a fluorescence change. The popular conception is that ATP causes a conformational change in the 44/62 complex such that the 62 protein is better positioned for interaction with the 45 protein (3). The mechanism in Fig. 7 uses ATP binding to the 44/62 complex to cause the conformational change that exposes the 62 protein for interaction with the 45 protein. ATP hydrolysis within the 45–44/62 complex causes an observable fluorescence change that may represent the opening of the 45 protein. As shown in Fig. 7 our results suggest that the ATP-dependent 45–44/62 complex can be associated with DNA without ATP hydrolysis. In the model (Fig. 7), this ATP hydrolysis independent DNA-45–44/62 complex represents a form of the 45 protein that does not have DNA through the center of the protein ring (nonconcatenated form). Since ATP binding must occur prior to ATP hydrolysis, it is plausible that the 44/62 complex chaperones the 45 protein to the primer template before the 45 protein ring is opened. The opening of the protein ring on DNA would enable the DNA to enter the center of the 45 protein. The ring opened 45 protein-44/62 complex on DNA may decay to a thermodynamically 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 ATPγS (23) and are thought to possess different roles in holoenzyme formation (23, 24, 25). The existence of a clamp loader-clamp intermediate has been shown in this system using surface plasmon resonance (24). This intermediate displayed similar ATP requirements to that of the 44/62–45 complex observed here in that it was dependent upon ATP hydrolysis for its function (24).

In conclusion, the fluorescence data presented here are highly suggestive of a mechanism in which the 44/62 complex mediates the association of the 45 protein with DNA in an ATP-dependent manner but without ATP hydrolysis. Upon ATP hydrolysis a conformational change is invoked in the 45 protein by the 44/62 complex that results in the functionally loaded 45 protein on DNA.

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Protein-Protein and Protein-DNA Interactions at the Bacteriophage T4 DNA Replication Fork: CHARACTERIZATION OF A FLUORESCENTLY LABELED DNA POLYMERASE SLIDING CLAMP

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