Dual Role of the 44/62 Protein as a Matchmaker Protein and DNA Polymerase Chaperone during Assembly of the Bacteriophage T4 Holoenzyme Complex†

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Received August 31, 1995; Revised Manuscript Received November 9, 1995⊗

ABSTRACT: Processive DNA synthesis in the bacteriophage T4 system requires the formation of a holoenzyme complex composed of the T4 DNA polymerase and the 44/62 and 45 accessory proteins. While ATP hydrolysis by the 44/62 protein is essential for holoenzyme formation, the role of the sliding clamp or processivity factor is attributed to the 45 protein. Beyond the need for ATP hydrolysis, the exact role of the 44/62 protein in complex assembly has not been clearly defined. In this paper, we have investigated the kinetics of complex assembly in the presence of both saturating and substoichiometric concentrations of the 44/62 protein. Under saturating conditions, complex assembly is 100% efficient, with all of the polymerase bound in a processive complex. Under conditions of limiting 44/62 protein, the 44/62 protein can act catalytically to assemble the 45 protein and polymerase into a productive complex. However, kinetic simulations indicate that a significant fraction of polymerase is sequestered in a nonproductive complex with the 45 protein. Thus, a second role for the 44/62 protein during complex assembly is that of a chaperone protein to ensure productive pol·45·DNA complex formation. We have also investigated the stability of the 45 protein on the DNA. The off rate of 0.003 s^{-1} for the 45 protein closely parallels that of the holoenzyme complex. Therefore, disassembly of the complex appears to involve the coordinated dissociation of both the 45 protein and the polymerase from the DNA.

In the bacteriophage T4 system, a processive DNA replication holoenzyme can be reconstituted from five phage proteins: the T4 DNA polymerase (43 protein), the 44/62 and 45 accessory protein complexes, and the single-stranded binding protein (32 protein) [reviewed in Young et al. (1992)]. The T4 DNA polymerase itself possesses both 5′−3′ polymerase activity as well as a 3′−5′ exonuclease (proofreading) activity, but its processivity depends upon the presence of the 44/62 and 45 accessory proteins. The 44/62 protein is a DNA-dependent ATP/dATPase that exists as a 4:1 complex of the 44 and 62 proteins, respectively (Jarvis et al., 1989a,b; Mace & Alberts, 1984; Huang et al., 1981). The homotrimeric 45 protein greatly enhances the ATPase activity of the 44/62 protein and, together with the 44/62 protein, forms a protein sliding clamp that is necessary for the assembly and maintenance of a processive replication complex.

Replication proteins whose functions are analogous to those of the 44/62 and 45 accessory proteins are found in both the *Escherichia coli* DNA polymerase III and the eukaryotic DNA polymerase δ systems. A recurring theme exists in all of these systems; a DNA polymerase (T4 43 protein, *E. coli* pol[1] III, eukaryotic pol δ), an ATPase [T4 44/62, *E. coli* γ complex, eukaryotic RF-C), and a clamp protein [T4 45 protein, *E. coli* β protein, eukaryotic PCNA (proliferating cell nuclear antigen)] are necessary for processive synthesis [reviewed in Stillman (1994)]. The crystal structures for the β protein (Kong et al., 1992) and PCNA (Krishna et al., 1994) reveal a striking feature of the clamp proteins. Despite mass differences of the monomer subunits, the dimeric β protein and the trimeric PCNA both adopt a ring structure around duplex DNA behind the primer/template junction. This unique topology of the clamp proteins allows for tight yet nonspecific binding to the DNA substrate, necessary requirements for a fast, processive DNA polymerase complex. The T4 45 protein is expected to exhibit a similar structure.

While ATP hydrolysis by the 44/62 protein is required during complex assembly, its role in elongation is still in dispute. Footprinting (Munn & Alberts, 1991b) and photocross-linking (Capson et al., 1991) experiments fail to provide clear evidence for the presence of the 44/62 protein in complex with polymerase and 45 protein on DNA, although these results could be as easily interpreted as a conformational change within the complex assembly rather than its dissociation that shifts 44/62 protein away from direct contact with the DNA. The size of the structures viewed by cryoelectron microscopy indicates that, in the absence of polymerase, 44/62 protein can load 45 protein onto nicked plasmid DNA and that 45 protein alone was seen to translocate away from the original loading site (Gogol et al., 1992). In addition, polymerase and 45 protein in high concentrations can form a processive complex on short linear DNA in the absence of 44/62, implying that the 45 clamp can even load without ATP hydrolysis (Reddy et al., 1993). Lastly, under conditions of limiting 44/62 protein, the amount of processive complex assembled exceeded that of the 44/62 protein concentration, indicating that the 44/62 protein

† This work was supported in part by National Institutes of Health Fellowship GM15239 (B.F.K.) and National Institutes of Health Grant GM13306 (S.J.B.).

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

[1] Abbreviations: pol, polymerase; PCNA, proliferating cell nuclear antigen; TBE, Tris-HCl/borate/EDTA; EDTA, ethylenediaminetetraacetate, sodium salt; dNTP, deoxynucleotide triphosphate; ssDNA, single-stranded DNA; SA, streptavidin; ATPγS, adenosine 5′-O-(3-thiotriphosphate).

0006-2960/96/0435-1084$12.00/0

acts as a catalyst during formation of the holoenzyme complex (Kaboord & Benkovic, 1995).

In this paper, we have attempted to further explore the role of the 44/62 protein in holoenzyme complex assembly. Using a forked primer/template substrate containing a biotin/streptavidin protein block on the 3′ end of the template strand, we have demonstrated that 44/62 protein is required to load the 45 clamp protein during complex assembly and that it can do so catalytically, implying that, once the complex is assembled, the 44/62 protein is not an essential component of the elongating complex. Thus, the 44/62 protein operates as a matchmaker protein (Sancar & Hearst, 1993) during DNA polymerase holoenzyme formation. Furthermore, we have discovered an additional role of the 44/62 protein in complex assembly in which it acts as a polymerase chaperone to ensure productive polymerase·45 association on the DNA. Finally, we show that the dissociation of the holoenzyme complex (consisting of polymerase and the 45 protein) features the coupled departure of both proteins from the DNA forked primer/template substrate.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized by Operon Technologies. The 3′-biotin incorporated into the template strand by Operon was the BioTEG derivative manufactured by Glen Research. [γ-^{32}P]ATP was purchased from New England Nuclear, and all nonradioactive deoxynucleotides were obtained from Pharmacia (Ultrapure). T4 polynucleotide kinase was from U.S. Biochemicals. The T4 exonuclease-deficient DNA polymerase (D219A) (Frey et al., 1993) was a generous gift of Dr. Michelle West Frey and Dr. Nancy Nossal (NIH). All references to the T4 DNA polymerase in the experiments presented in this paper refer to the D219A exo$^-$ polymerase mutant. The 44/62 and 45 accessory proteins were purified from overproducing strains obtained from Dr. William Konigsberg (Yale University). The concentrations of 44/62 and 45 proteins are reported in terms of 4:1 ratio of complex and trimer, respectively (Jarvis et al., 1989).

Primer/Template Construction. All oligonucleotides were gel purified as described by Capson et al. (1992). The template strand of the biotin-labeled duplex (Bio34/62) was constructed by ligation of two shorter oligonucleotides (3′-biotin 23mer and a 39mer) as described in Kaboord and Benkovic (1993, 1995). The duplex was gel purified on a nondenaturing gel, quantitated by enzymatic assay, and 5′-^{32}P-labeled using polynucleotide kinase as previously described (Capson et al., 1992). Construction of the forked primer/template (Bio34/62/36) was completed by hybridization of a 36mer (in 5% excess) to the Bio34/62 duplex.

Methods. All rapid quench experiments were performed at 20 °C on the instrument described by Johnson (1986). The assay buffer used in all of the complex assembly experiments consisted of 25 mM Tris-OAc (pH 7.5), 150 mM KOAc, 10 mM $Mg(OAc)_2$, and 10 mM 2-mercaptoethanol. All reagent, substrate, and protein concentrations listed are final reaction concentrations. Reactions were quenched manually by addition of 2 M HCl. The quenched samples were immediately extracted with phenol $CHCl_3$ (1:1) and neutralized with 3 M NaOH in 1 M Trizma base to prevent acid hydrolysis of the DNA products. Polymerization reaction products were analyzed on 16% sequencing gels as described (Kaboord & Benkovic, 1993) in which the top reservoir running buffer was heated to 80−85 °C prior to sample loading. Gel images were obtained and quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software v3.3.

Titration of the 45 Protein. Assembly of the T4 holoenzyme complex was examined in the presence of limiting concentrations of the 45 protein. A solution of 500 nM Bio34/62/36, 550 nM streptavidin, 1 mM ATP, and 10 μM dCTP was mixed with 100 nM T4 D219A polymerase, 550 nM 44/62 protein, and 45 protein (10−550 nM) and incubated for varying times (0.25−60 s) in the rapid quench instrument. DNA synthesis by the assembled complex was initiated by introduction of the remaining dNTPs (10 μM each) and single-stranded salmon sperm DNA trap (1 mg/mL) via the third syringe. Reactions were quenched manually after 10 s by addition of HCl as described above.

Titration of the 44/62 Protein. Assembly of the T4 holoenzyme complex was examined under limiting 44/62 protein concentrations. A solution of 500 nM Bio34/62/36, 550 nM streptavidin, 1 mM ATP, and 10 μM dCTP was mixed with 100 nM T4 D219A polymerase, 550 nM 45 protein, and 44/62 protein (0−550 nM) and incubated for varying times (0.25−60 s) in the rapid quench instrument. DNA synthesis by the assembled complex was initiated by introduction of dNTPs and ssDNA trap and quenched as described above.

Enzyme Spike Experiment. Complex was assembled using limiting 44/62 protein by mixing a solution of 500 nM Bio34/62/36, 550 nM streptavidin, 1 mM ATP, and 10 μM dCTP with a solution containing 500 nM D219A polymerase, 10 nM 44/62 protein, and 550 nM 45 protein (concentrations of all reactants are final concentrations). Aliquots were removed at various times (5−120 s) and added to tubes containing 10 μM dNTPs and 1 mg/mL single-stranded DNA trap. Each reaction was quenched with the addition of 2 M HCl followed by phenol chloroform extraction and neutralization with 3 M NaOH. In an identical reaction, a second aliquot of polymerase (100 nM) was added to the original assembly reaction after 30 s (time at which the assembly reaction normally plateaus). Primer extension products were analyzed as described above.

Complex Assembly in the Presence of ATPγS. As a prelude to the 45 protein k_{off} determination, the use of ATPγS as a competitor for the ATP/dATP pool present in the complex assembly experiment was evaluated. A solution of 200 nM Bio34/62/36, 220 nM streptavidin, 50 μM ATP, 10 μM dNTPs, and ATPγS (1, 2, or 4 mM) in assay buffer was mixed with a solution composed of 100 nM D219A polymerase, 220 nM 44/62 protein, and 220 nM 45 protein in assay buffer at room temperature (∼21 °C). Aliquots were removed at 10, 20, 30, and 60 s, quenched into 1 M HCl, phenol chloroform extracted, and neutralized with 3 M NaOH as described above. DNA products were analyzed on a 16% sequencing gel and quantitated as described above. The purity of the ATPγS stock was 98% as determined by NMR.

Determination of k_{off} for 45 Protein. Four parallel reactions were set up in which a solution of ^{32}P-labeled Bio34/62/36mer in assay buffer plus 0.5 mg/mL bovine serum albumin (BSA) was incubated with 40 μL of streptavidin agarose slurry (Sigma) for 30 min at room temperature with gentle mixing. The resin was pelleted in a microfuge for 30 s and washed with assay buffer plus 0.25 mg/mL BSA

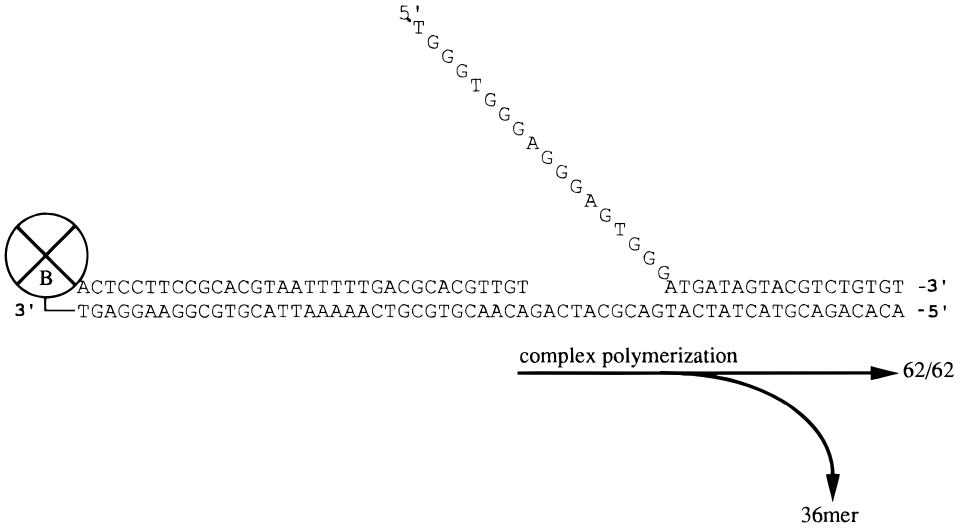


FIGURE 1: Sequence of the biotinylated forked primer/template, Bio34/62/36. DNA synthesis by the complex results in extension of the primer (34mer) to a 62mer, displacing the fork strand (36mer) in the process. Primer extension by the polymerase alone terminates after the 10-base pair gap is filled. B is the biotin derivative incorporated into the template strand. The quartered circle represents the bound streptavidin tetramer.

three times until no appreciable counts were found in the supernatant. The DNA streptavidin agarose pellet was resuspended in assay buffer containing 1 mM ATP, 200 nM D219A polymerase, 550 nM 44/62 protein, 550 nM 45 protein, and 0.25 mg/mL BSA (all concentrations are final in a 100 μL reaction volume). The mixture was incubated at room temperature for 1 min to allow for complex assembly on the bound DNA. For the time zero point, dNTPs (50 μM) and ssDNA trap (1 mg/mL) were added to the mixture and the mixture was incubated for an additional 10 s. The reaction was quenched by the addition of EDTA (0.33 M final concentration). For the three remaining reactions, the protein DNA complexes were incubated in the presence of ssDNA trap (1 mg/mL) for 7, 15, and 30 min, during which the resin was washed three times with assay buffer plus 0.25 mg/mL BSA to remove any dissociated proteins (7 min was the minimum time required to perform the three washes). The resin pellet was then resuspended in a solution of assay buffer, 1 mM ATP, 200 nM D219A polymerase, and 550 nM 44/62 protein and incubated for 1 min to assemble complexes with any 45 protein still bound to the immobilized DNA. ssDNA trap (1 mg/mL) and dNTPs (50 μM) were added to initiate DNA synthesis. The reactions were quenched after 10 s with EDTA as described above. All DNA-resin pellets were washed with TE [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA] to dilute the high concentration of EDTA for better gel resolution. The pellets were resuspended in 50 μL of TE plus 50 μL of formamide load buffer and heated to $\geq$90 °C for 5 min to release the Bio34/62/36mer from the streptavidin agarose. A fraction of the supernatants was loaded onto a 16% acrylamide/8 M urea/TBE sequencing gel to analyze the amount of strand displacement synthesis. A parallel set of reactions were conducted in which the entire experiment was done in the absence of accessory proteins to account for any extension products >44mer that were due to synthesis by polymerase alone on DNA substrates from which the 36mer had been stripped during the wash steps. The amounts of 61+62mer products in the control (polymerase) reactions were subtracted from the total.

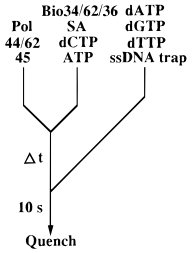


FIGURE 2: Schematic of the complex assembly experiment used in the 45 and 44/62 protein titrations. The three syringes of the rapid quench were loaded with the indicated solutions (see text for concentrations) and the contents mixed according to the flow scheme shown.

Kinetic Simulations. The simulations of the kinetic data were performed using the program KINSIM (Barshop et al., 1983) as modified by Anderson et al. (1988).

RESULTS

Assembly of the Complex. The T4 DNA replication holoenzyme can be assembled stoichiometrically on the forked primer/template, Bio34/62/36 (Figure 1) (Kaboord & Benkovic, 1995). Key to the successful assembly of the complex on this substrate is the presence of a biotin derivative incorporated synthetically onto the 3′ end of the template strand. Incubation of the substrate with streptavidin results in a protein block that prevents translocation of the mobile accessory proteins off the end of the DNA. Bidirectional translocation away from the primer/template junction is further impeded by the presence of the 36mer fork strand annealed to the 5′ end of the template strand. DNA synthesis by the complex results in displacement of the 36mer strand to produce a fully elongated primer (mixture of 61- and 62mer). Since the polymerase alone cannot perform strand displacement synthesis, its products ($\leq$44mer) are easily distinguishable from those of the complex.

A schematic representation of the experiment used for studying the assembly of the holoenzyme complex is shown in Figure 2. A solution of Bio34/62/36 (500 nM), streptavidin, ATP, and dCTP (the first deoxynucleotide to be incorporated) is mixed with a solution containing the D219A

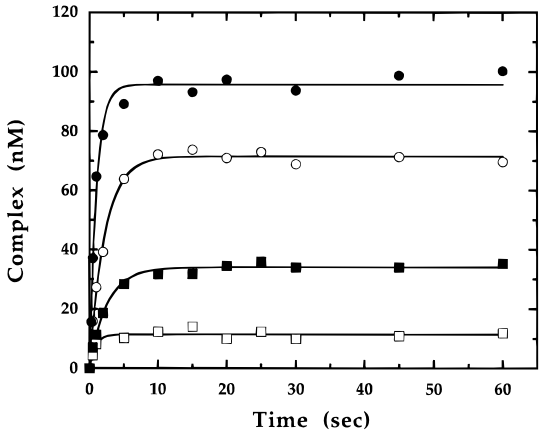


FIGURE 3: Effect of the 45 protein titrations on complex assembly. The complex was assembled by mixing 500 nM Bio34/62/36, 550 nM 44/62 protein, 100 nM T4 DNA polymerase, and 25 nM (□), 50 nM (■), 100 nM (○), and 550 nM (●) 45 protein as shown in Figure 2 for the indicated times. DNA synthesis by the assembled complexes was initiated by the addition of dNTPs and single-stranded DNA trap. Each time course was fit to a single exponential equation.

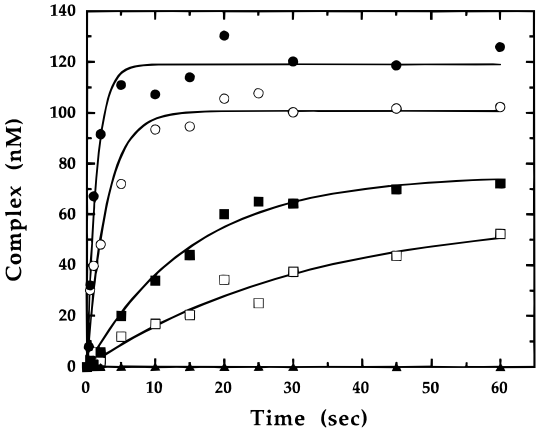


FIGURE 4: Effect of the 44/62 protein titrations on complex assembly. The complex was assembled by mixing 500 nM Bio34/62/36, 550 nM 45 protein, 100 nM T4 DNA polymerase, and 0 nM (▲), 2 nM (□), 10 nM (■), 50 nM (○), and 550 nM (●) 44/62 protein as shown in Figure 2 for the indicated times. DNA synthesis by the assembled complexes was initiated by the addition of dNTPs and single-stranded DNA trap. Each time course was fit to a single exponential equation.

polymerase (100 nM) and 44/62 and 45 accessory proteins (550 nM each) for varying times in the rapid quench instrument to allow for the assembly of the protein complex on the DNA. Since the accessory proteins are saturating with respect to the DNA, in time all of the DNA should be populated with accessory proteins. Therefore, the amount of complex formed reflects the polymerase concentration (which is limiting) unless specified otherwise. Thus, the initial mixing represents a competition between accessory proteins and polymerase for the primer/template junction. In the presence of saturating accessory proteins, eventually all of the polymerase will be sequestered into a poised complex due to the slow k_{off} (0.002 s^{-1}) of the complex relative to that of the polymerase (Kaboord & Benkovic, 1995). A solution containing the remaining dNTPs and single-stranded DNA (to trap any free proteins) is introduced to initiate DNA synthesis by any assembled complexes. Manual addition of HCl is used to quench the reactions.

Titration of the 45 Protein. Since the 45 protein constitutes the sliding clamp in the T4 DNA replication system (Reddy et al., 1993; Gogol et al., 1992; Kaboord & Benkovic, 1995), decreasing the concentration of this protein in the complex assembly reaction should result in a corresponding decrease in the amount of complex formed and the rate of its formation. Figure 3 shows the results from a titration experiment in which the 45 protein was present in limiting concentrations in the assembly reaction. As the concentration of 45 protein is decreased, there is a proportional decrease in the amount of complex formed which is to be expected since the 45 protein is an essential component. The fact that the amount of complex assembled is approximately 20% less than the 45 protein concentration (determined spectrophotometrically) most likely reflects an ambiguity in the active protein concentration due to the presence of contaminants in the 45 protein preparation (purity estimated at 90%) and/or a shift in the monomer−trimer equilibrium, especially at the lowest concentration where 25 nM 45 protein produces only 15 nM complex (60% of the 45 protein concentration).

Titration of the 44/62 Protein. Systematic titrations of the 44/62 protein in the complex assembly reaction were also performed in which the concentration of 44/62 protein was decreased while the concentration of 45 protein and polymerase were held constant at 550 and 100 nM, respectively. In contrast to the 45 titrations, decreasing the amount of 44/62 protein to substoichiometric concentrations resulted in the formation of quantities of complex greater than the concentration of 44/62 protein present (Figure 4). This is most evident in the cases of 2 nM 44/62 producing 52 nM complex and 10 nM 44/62 forming 70 nM complex. These data agree with the 44/62 protein playing a catalytic role in the assembly of a processive leading strand complex. The concentration dependence of 44/62 protein on complex formation was calculated by fitting the curves in Figure 4 to a single exponential equation and replotting the rates as a function of 44/62 concentration to obtain an apparent second order rate constant of 7×10^6 M^{-1} s^{-1}. Closer examination of the kinetics of complex formation under limiting 44/62 protein conditions reveals that, while this protein acts catalytically, the reactions never achieve 100% complex formation (100 nM). Instead, the assembly reactions reach a premature plateau, reflecting a termination of assembly that does not occur in the presence of higher (550 nM) 44/62 protein concentrations. Kinetic simulations show that the plateau does not represent an equilibrium between complex formation and dissociation (see Discussion).

Investigation of the Assembly Kinetics in the Presence of Limiting 44/62 Protein. The premature termination of complex assembly in the 44/62 protein titration experiment suggests that there is a loss or sequestration of a necessary component of the complex. The plateaus seen are characteristic of a reaction containing the accessory proteins since all of the DNA can be turned over by the polymerase alone to form 44mer product (data not shown). This also verifies the presence of the 36mer fork strand on every primer/template duplex, demonstrating that each DNA construct should be a valid substrate for complex assembly. To determine whether the polymerase was somehow being sequestered, an assembly reaction was performed in which 44/62 protein was limiting at 10 nM with polymerase and 45 protein present at 100 and 550 nM, respectively, conditions identical to those previously shown in Figure 4 (closed squares). However, at the point in time where the reaction

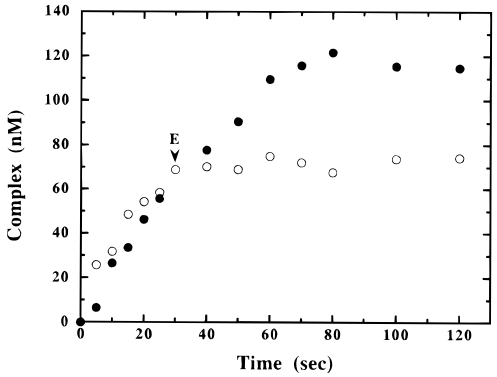


FIGURE 5: Effect of additional polymerase on the amount of complex assembled in the presence of limiting 44/62 protein. The complex was assembled by mixing 500 nM Bio34/62/36, 100 nM T4 DNA polymerase, 550 nM 45 protein, and 10 nM 44/62 protein, and aliquots were removed at the indicated times and added to dNTPs and ssDNA trap to initiate DNA synthesis (○). In an identical reaction, additional polymerase (100 nM) was added to the assembly reaction at 30 s (●).

curve normally reaches a plateau (30 s), an additional aliquot of polymerase (100 nM) was added to the assembly reaction mixture (Figure 5). Upon addition of the second aliquot of polymerase, another equivalent of complex was formed (relative to the first). Thus, the plateau in the reaction could be overcome upon addition of polymerase, demonstrating that there were sufficient accessory proteins poised for complex formation but polymerase was missing or inactivated. Since the amount of polymerase sequestered exceeds the concentration of 44/62 protein present in the reaction mixture, the nonproductive complex must consist of polymerase bound to 45 protein.

Dissociation Constant, koff, of the 45 Protein. Since the 45 protein acts as the sliding clamp in the processive complex and, by analogy to the β protein and PCNA, is believed to encircle the DNA, it is interesting to speculate what the lifetime of the 45 protein on the streptavidin Bio34/62/36mer substrate would be. Difficulties arose in the initial attempts to preload the 45 protein onto SA-Bio34/62/36 and measure the amount bound as a function of time because the ATP pool must be rapidly and completely depleted to prevent reloading of 45 onto DNA by 44/62. The problem encountered was related to the need to verify the presence of the 45 protein on DNA by the strand displacement assay. During this time, there was a large amount of dATP-mediated complex formation by the 44/62 protein (data not shown). Therefore, even if the ATP pool could be rapidly diminished by simple dilution or hydrolysis, dATP was always reintroduced with dNTPs and polymerase during the displacement assay on this DNA substrate.

In an effort to circumvent this problem, ATPγS was introduced during the time course to compete with both ATP and dATP for the ATP-binding site of the 44/62 protein. In a control experiment, a solution containing DNA, dNTPs, ATP, and ATPγS at a 20-, 40-, and 80-fold excess over ATP was mixed with a solution of polymerase and 44/62 and 45 proteins for 0−60 s and the reaction quenched with HCl. Successful inhibition by ATPγS should have prevented any complex formation. However, by 10 s (minimum time required to perform a manual quench of the strand displacement assay), 70% of the potential complexes was formed even in the presence of an 80-fold molar excess of ATPγS over ATP (data not shown). This was surprising in that

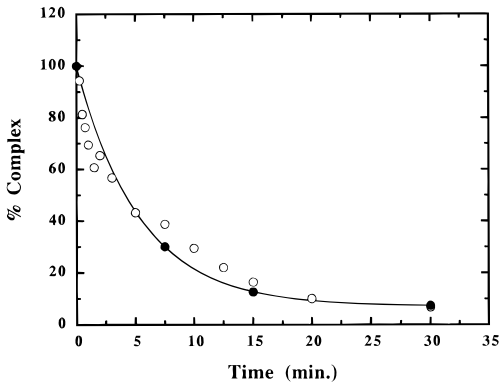


FIGURE 6: Determination of the k_{off} for the 45 protein. Complexes were assembled on Bio34/62/36 immobilized on streptavidin−agarose and allowed to decay for the indicated times, after which polymerase, 44/62, and ATP were added back to determine if any 45 protein remained bound after polymerase dissociation from the complex. The amount of complex formed under these conditions (●) was superimposed on the original complex k_{off} data (○) from Kaboord & Benkovic (1995). The 45 k_{off} data were fit to a decreasing exponential equation with a floating end point (solid line).

kinetic simulations using the reported K_I for ATPγS of 50 μM and $K_{M\ ATP}$ of 150 μM (Piperno et al., 1978; Bedinger & Alberts, 1983; Jarvis et al., 1989b) indicate that ≤1.5% of the 44/62·ATP complex should exist.

An alternative approach would be to measure the k_{off} of the 45 protein after its participation in a processive complex. The holoenzyme complex was assembled on Bio34/62/36mer bound to streptavidin agarose rather than soluble streptavidin. Assembled complexes were precipitated, resuspended, and allowed to decay with time. Dissociated proteins, ATP, and nonspecifically associated proteins were removed by centrifugation of the resin and removal of the supernatant periodically during the decay time. Polymerase, 44/62 protein, and ATP were added back to the resin to reassemble a complex using any 45 protein remaining bound to the immobilized DNA, and the complexes were then quantitated by their ability to perform strand displacement synthesis in the presence of dNTPs and ssDNA trap. Figure 6 shows that the loss of bound 45 protein at a rate of 0.003 s^{-1} closely parallels the k_{off} of the complex previously measured (0.002 s^{-1}) (Kaboord & Benkovic, 1995). Thus, the k_{off} of the complex most likely represents the simultaneous dissociation of the polymerase and the 45 protein. The k_{off} of the T4 45 protein significantly differs from the ≥6-fold slower off rates observed for the β subunit of *E. coli* (Stukenberg et al., 1994) and eukaryotic PCNA (Podust et al., 1995).

Kinetic Simulations of the 44/62 Protein Titration Data. The complex assembly reactions reflect a binding competition between free polymerase and accessory proteins for the primer/template junction. Since the first dNTP to be incorporated (dCTP) is present during this incubation, any polymerase molecules that bind to the primer terminus in the absence of accessory proteins will incorporate the single nucleotide to form a 35mer. Because of its distributive nature (k_{off} = 3 s^{-1}; B. F. Kaboord, unpublished results), the polymerase will dissociate and again be faced with the possibility of binding alone or with accessory proteins to form a stable complex. Any polymerase molecules that bind to the primer terminus with accessory proteins will also produce a 35mer but will remain bound (k_{off} = 0.002 s^{-1}; Kaboord & Benkovic, 1995). Upon addition of the remain-

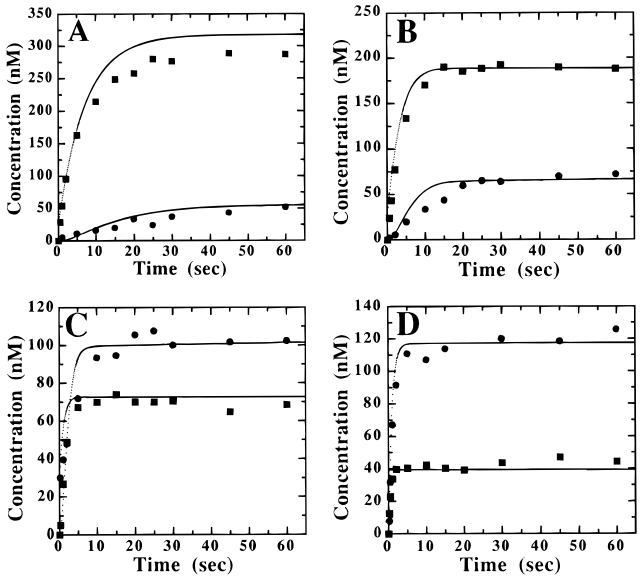


FIGURE 7: Kinetic simulations of the 44/62 protein titrations. The amounts of complex (●) and 35mer (■) produced during the assembly incubations in the presence of varying concentrations of the 44/62 protein were fit to a computer simulation based on the mechanism depicted in Scheme 1. Complex assembly was quantitated by measuring the amount of strand displacement synthesis (61- and 62mer bands) upon introduction of dNTPs and ssDNA trap following the initial assembly incubation. Concentrations of 44/62 protein used in the titrations were 2 nM (A), 10 nM (B), 50 nM (C), and 550 nM (D).

ing dNTPs and trap, the 35mer is fully elongated (61- and 62mer). Therefore, the more 35mer remaining after introduction of dNTPs and trap, the more free polymerase molecules had visited the primer/template junction which, in turn, reflects the increased length of time required to form a complex under limiting accessory protein concentrations.

Figure 7 shows the data for complex and 35mer production for each concentration of 44/62 protein examined in the titration experiment. Recall that the amount of complex assembled is determined by quantitation of the amount of strand displacement products (61- and 62mer) while the 35mer is the residual product of the polymerase acting alone.

Table 1: Kinetic Parameters for the Complex Assembly Mechanism (Scheme 1)[a]

step	k_{+n}	k_{-n}
1	$1-2\times10^7$ M^{-1} s^{-1}	13 s^{-1}
2	1×10^8 M^{-1} s^{-1}	0.003 s^{-1}
3	13 s^{-1}	$1-2\times10^7$ M^{-1} s^{-1}
4	1×10^8 M^{-1} s^{-1}	0.002 s^{-1}
5	500 s^{-1}	0.1 s^{-1}
6	1×10^8 M^{-1} s^{-1}	0.002 s^{-1}
7	2×10^{16} M^{-2} s^{-1}	13 s^{-1}
8	13 s^{-1}	$1-2\times10^7$ M^{-1} s^{-1}
9	$0.9-1\times10^8$ M^{-1} s^{-1}	2 s^{-1}
10	50 s^{-1}	0.1 s^{-1}
11	2 s^{-1}	1×10^8 M^{-1} s^{-1}
12	$2-2.5\times10^8$ M^{-1} s^{-1}	1−1.3 s^{-1}
13	$2-2.5\times10^8$ M^{-1} s^{-1}	1−1.3 s^{-1}

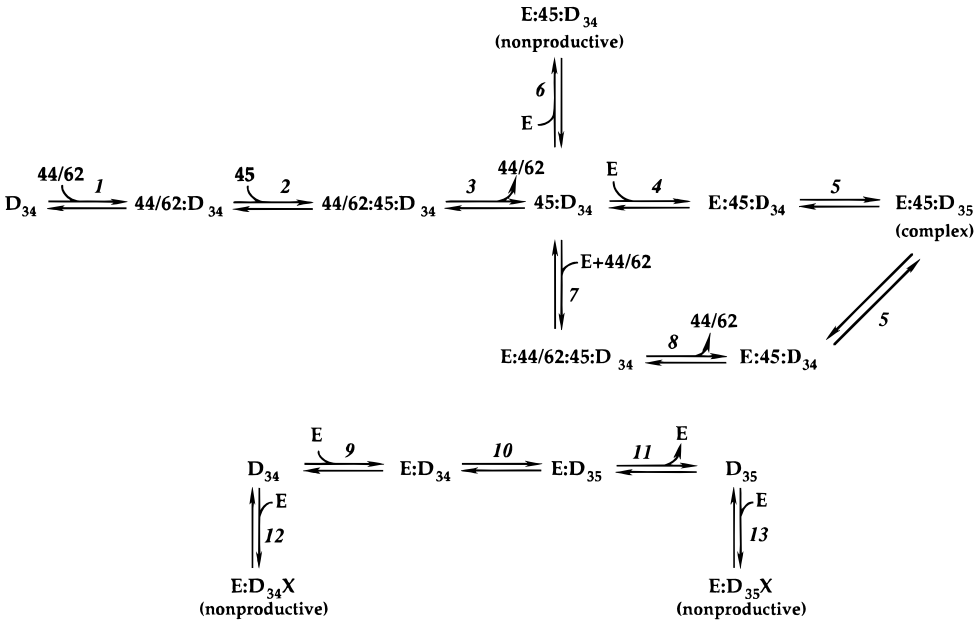
[a] All second order rate constants are assumed to be diffusion-limited. Constants determined experimentally include k_{-2}, k_{-4}, k_{+5}, k_{-5}, k_{-9}, k_{+10}, k_{-10}, and k_{+11}. Values for steps 1, 3, 7, and 8 were determined by simulation on the basis of the K_m value of 1.3 μM of 44/62 for DNA (3′ ends primer/template) (Jarvis et al., 1989b). The constants listed for steps 6, 12, and 13 were determined by simulation.

These data were simulated using the program KINSIM, the mechanism shown in Scheme 1, and the kinetic parameters listed in Table 1. The mechanism takes into account the catalytic role of the 44/62 protein in complex assembly (steps 1−3) as well as the competition between free polymerase (step 9) and complex for the primer/template junction. In addition, the polymerase can associate with the 45 protein clamp productively (step 4) or nonproductively (step 6). There are two means of forming productive complex, one that involves independent binding of polymerase to 45 protein (step 4) and one that is dependent upon 44/62 protein concentration (step 7). This additional role of the 44/62 protein in complex assembly will be further described in the Discussion.

DISCUSSION

Loading of the Sliding Clamp (45 Protein). We have shown that, during assembly of the T4 DNA holoenzyme complex, the 44/62 accessory protein serves as a matchmaker

Scheme 1



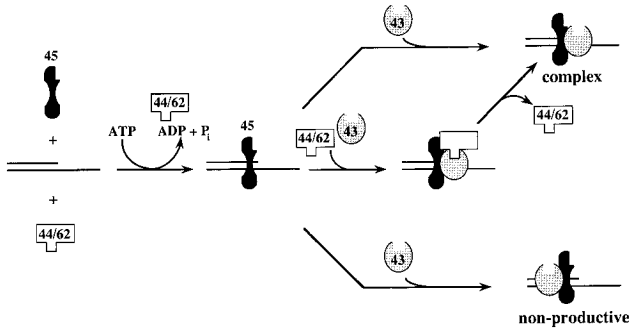


FIGURE 8: Model showing the dual role of the 44/62 protein in assembly of the T4 DNA holoenzyme complex. The 44/62 protein acts catalytically to load the 45 protein clamp onto the DNA substrate. Independently of the 44/62 protein, the polymerase has equal probability of binding to the 45 protein productively or nonproductively. Alternatively, the 44/62 protein can serve as a chaperone for the polymerase to ensure productive binding to the 45 protein and the assembly of a functional complex.

protein in loading the 45 protein onto the DNA (Figure 8). Catalytic amounts of 44/62 protein in the assembly reactions generated concentrations of processive complexes (defined by strand displacement synthesis) exceeding the 44/62 protein concentration by as much as 25-fold (Figure 4). The apparent second order rate constant of 7×10^6 M^{-1} s^{-1} for this process is inclusive of steps 1−3 of Scheme 1, reflecting 44/62 binding, 45 loading, and the subsequent recycling of the 44/62 protein, accounting for the difference between the calculated values and the simulated values (Table 1) which are closer to diffusion limits. The catalytic role of 44/62 is further substantiated by the experiment in which ATPγS was present during assembly of the complex at a 20- and 80-fold molar excess over ATP. On the basis of the reported K_I for ATPγS and K_M for ATP (Bedinger & Alberts, 1983; Piperno et al., 1978; Jarvis et al., 1989b), ≤1.5% 44/62·ATP complex should exist, yet this minor population was capable of approximately 20−60 turnovers, respectively, during the course of complex assembly. The catalytic role of the 44/62 protein in DNA replication complex assembly is consistent with its role as a matchmaker in assembly of transcription complexes (Tinker et al., 1994). Similarly, a single γ complex in the *E. coli* polymerase III system (functional homolog of the T4 44/62 protein) was determined to catalyze loading of the β protein (analogous to the T4 45 protein) onto both the leading and lagging strands during coordinated DNA synthesis at a replication fork (Stukenberg & O'Donnell, 1995) and has the potential to perform multiple loadings on the lagging strand (Stukenberg et al., 1991).

The first steps in Figure 8 depict the catalytic use of 44/62 protein in 45 loading as distinct events prior to the binding of polymerase in the complex. The way the assembly experiment is designed (Figure 2), all of the necessary proteins are mixed simultaneously with the DNA; thus, it is not immediately apparent as to the binding order of the various protein components during complex assembly. On the basis of footprinting (Munn & Alberts, 1991a) and cross-linking (Capson et al., 1991) evidence, it is known that the 44/62 and 45 accessory proteins can form a complex at the primer/template junction in the presence of ATPγS. Since ATP hydrolysis is required during assembly of the holoenzyme complex (Huang et al., 1978; Piperno & Alberts, 1978; Jarvis et al., 1991) and is required for cross-linking of the polymerase in the complex (Capson et al., 1991), it is easiest to envision that binding of the accessory proteins precedes binding of the polymerase. However, an alternative model may involve the 44/62 protein bringing the 45 protein and polymerase (43 protein) together simultaneously to form a DNA·44/62·45·43 quaternary complex, after which the 44/62 protein dissociates to catalyze the association of another 45·43 pair on another primer/template. Thus, there is no isolated 45 protein preloaded onto the DNA.

Several lines of evidence in the literature favor the first model in which 45 protein is preloaded onto the DNA and subsequent 45·43 protein interactions are not necessarily mediated by the presence of the 44/62 protein. First, the mobile component of the accessory protein complex, at least in the absence of polymerase, is believed to be the 45 protein alone (Gogol et al., 1992), indicating that 45 can be preloaded onto DNA and remain bound independently of the 44/62 protein. Second, 45 protein affinity columns were able to bind polymerase in the absence of the 44/62 protein (Formosa et al., 1983). Third, Reddy et al. (1993) have shown that a processive complex can assemble on linear DNA in the absence of 44/62 protein (albeit inefficiently). Last, the requirement for biotin/streptavidin blocks on both ends of the primer/template (Kaboord & Benkovic, 1993) or a combination of a biotin/streptavidin block and an annealed fork strand (Kaboord & Benkovic, 1995) to prevent translocation of the 45 protein off linear DNA, together with the k_{off} of 0.003 s^{-1} determined for the 45 protein, suggests that the mobile 45 protein resides on the DNA for a significant time prior to polymerase binding at diffusion limits. In the event that the 45 protein is not as stable prior to its involvement in complex formation, there is sufficient latitude in the 45 protein k_{off} (k_{-2} in Scheme 1) such that the simulations shown in Figure 7 would be insensitive to a change in this value over a range of 3 orders of magnitude. Additional evidence for polymerase binding to the 45 protein independently of the 44/62 protein is described below.

Once the 45 protein is loaded onto the DNA, its lifetime on the DNA parallels the lifetime of the processive complex previously determined (Kaboord & Benkovic, 1995; Hacker & Alberts, 1994). This suggests that the disassembly of the complex reflects the simultaneous dissociation of the 45 protein and polymerase from the DNA, with the rate of 45 dissociation possibly dictating the stability of the 45·polymerase·DNA complex. In this respect, the T4 system would contrast dramatically with the *E. coli* system in which the polymerase III holoenzyme partially disassembles, leaving the β clamp behind on the DNA (Stukenberg et al., 1994). The catalytic role of the 44/62 and β clamp loading proteins is necessary whether a new clamp protein is loaded or a recycled clamp (still associated with the holoenzyme complex) is relocated to the next Okazaki fragment.

44/62 Protein as a Chaperone in Complex Assembly. Once the 45 protein is loaded onto the DNA, its fate can be directed along three different routes (Figure 8 and Scheme 1). First, polymerase can bind to the DNA·45 binary complex independently of the 44/62 protein to form a processive (productive) holoenzyme complex (Scheme 1, step 4). Examination of the 44/62 titration experiment (Figure 4) reveals that as much as 52 nM complex is formed with only 2 nM 44/62 protein present. Comparison of the amount of complex formed in each curve to the 44/62 protein concentration and extrapolation to zero 44/62 protein predict

that approximately 50 nM complex (50% of total possible) would still be formed (in reality the absence of 44/62 protein would result in no complex formed on this substrate since the 45 protein could not be loaded). Therefore, polymerase must be able to bind to 45 protein independently of the 44/62 protein. Further examination of the 44/62 titration experiment shows that, in the presence of low concentrations of 44/62 protein, the curves plateau before reaching 100 nM (100%). This plateau does not represent an equilibrium between formation and dissociation of the complex. Simulation of this scenario indicates that the k_{off} for the complex is not a significant factor within the time limits of this experiment. Therefore, it is hypothesized that polymerase is somehow being sequestered nonproductively (step 6).

Evidence to support this theory is given by the data in Figure 5 in which the plateau in complex formation is alleviated by the addition of polymerase to the reaction mixture after 30 s (time at which the reaction normally reaches a plateau). Furthermore, inclusion of dCTP (the first dNTP to be incorporated) during assembly time (Figure 2, Δt) allows us to monitor the binding and dissociation of free polymerase prior to its assembly into a stable complex. Examination of the time courses for 35mer formation at substoichiometric 44/62 concentrations (Figure 7A,B) shows them reaching a plateau at the same time as the time courses for complex formation but short of turning over all of the DNA. Thus, if there was free polymerase in the reaction mixtures, the amount of 35mer produced would approach the total DNA concentration (minus the amount of productive complex formed). Since this does not occur, we conclude that polymerase must bind nonproductively with the 45 protein and, by simulation, must have a $t_{1/2}$ comparable to that of the functional complex. Furthermore, as noted above in the extrapolation to zero 44/62 protein levels, the polymerase partitions equally between productive and nonproductive complexes with the 45 protein. One interpretation is that these nonproductive complexes may reflect a sidedness to the 45 protein similar to that observed for the β protein (Kong et al., 1992) but with the polymerase able to bind either side of the 45 protein. A second interpretation is that the polymerase is bound to the proper face of the 45 protein but is not oriented correctly with respect to the 3′ OH primer terminus. These nonproductive complexes are depicted in Figure 8, for simplicity's sake only, as polymerase binding to the wrong side of the 45 protein. Whether this nonproductive association occurs in solution or on the DNA cannot be concluded from the available data.

Perhaps the most interesting aspect of the 44/62 protein titration data is that, in the presence of ≥50 nM 44/62 protein, all of the polymerase is partitioned into productive complexes. Thus, we conclude that there is another role for 44/62 protein in holoenzyme complex assembly, namely, one of a polymerase chaperone, ensuring efficient binding of polymerase to the DNA·45 (steps 7 and 8). Therefore, the 44/62 concentration dependence manifests itself not only in the rate of 45 protein loading but also in the final amount of functional complex assembled. Independent of the scenario of how the pol·45 nonproductive complexes arose, it is clear that some degree of structural organization within the complex must be mediated by the 44/62 protein. The nonproductive DNA·45·43 complexes are probably an artifact of the in vitro system under these conditions since the 44/62 and 45 proteins are more equivalently expressed in vivo. However, the observation of nonproductive complexes in this system allowed us to identify the role of the 44/62 protein as a chaperone in complex assembly.

Steps 9−13 in Scheme 1 delineate the side reactions of binding and turnover by the polymerase alone during complex assembly. Simultaneous introduction of all the proteins in the reaction inevitably results in a competition between polymerase and accessory proteins for the primer/template junction (Kaboord & Benkovic, 1993). On this forked primer/template, two additional steps involving nonproductive binding of the polymerase to either Bio34/62/36mer or Bio35/62/36mer (steps 12 and 13, respectively) were found through simulations to be necessary for attenuation of the rate of 35mer production during the assembly reaction. Without these steps, the amount of 35mer produced by simulation exceeds the levels actually produced experimentally and at a much faster rate. Most likely, these steps represent binding of the polymerase to other portions of the DNA substrate (Capson et al., 1992; Kaboord & Benkovic, 1995). One of the roles of the accessory proteins is to prevent this nonspecific binding (Jarvis et al., 1989b).

Summary. In this paper, we have outlined two roles of the T4 44/62 protein in assembly of the holoenzyme complex. First, the 44/62 protein can serve catalytically in the loading of the 45 protein clamp onto DNA. Second, 44/62 protein acts as a chaperone in the subsequent binding of polymerase to the DNA·45 complex to complete the formation of a processive complex. While it is known that ATP hydrolysis is involved in complex assembly, ongoing studies in our laboratory indicate that the chaperone function of the 44/62 protein is ATP-independent (Anthony Berdis, personal communication). In addition to determining the function of the 44/62 protein in holoenzyme assembly, we have shown that dissociation of the pol·45 holoenzyme complex occurs by a coordinated mechanism involving the simultaneous release of both proteins from the primer/template.

Because of the remarkable functional similarities between the accessory proteins of the bacteriophage T4, *E. coli* polymerase III, and eukaryotic polymerase δ systems, it will be interesting to determine if the mechanistic features described in this paper for T4 holoenzyme complex assembly compare or contrast with the leading and lagging strand complexes of these other systems.

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BI9520747