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DNA-Induced Switch from Independent to Sequential dTTP Hydrolysis in the Bacteriophage T7 DNA Helicase

Donald J. Crampton,[1] Sourav Mukherjee,[1] and Charles C. Richardson[1,*]
[1] Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
Boston, Massachusetts 02115

Summary

We show that the mechanisms of DNA-dependent and -independent dTTP hydrolysis by the gene 4 protein of bacteriophage T7 differ in the pathways by which these reactions are catalyzed. In the presence of dTTP, gene 4 protein monomers assemble as a ring that binds single-stranded DNA and couples the hydrolysis of dTTP to unidirectional translocation and the unwinding of duplex DNA. When mixing wild-type monomers with monomers lacking the catalytic base for the dTTPase reaction, we observe that each wild-type subunit hydrolyzes dTTP independently in the absence of single-stranded DNA. Conversely, when either these catalytically inactive monomers or altered monomers incapable of binding single-stranded DNA are mixed with wild-type monomers, a small fraction of altered to wild-type monomers causes a sharp decline in DNA-dependent dTTP hydrolysis. We propose that sequential hydrolysis of dTTP is coupled to the transfer of single-stranded DNA from subunit to adjacent subunit.

Introduction

Helicases are motor proteins that translocate unidirectionally along single-stranded nucleic acids using energy derived from nucleotide hydrolysis (Lohman and Bjornson, 1996; Matson and Kaiser-Rogers, 1990; Patel and Picha, 2000). The gene 4 protein encoded by bacteriophage T7, a helicase that unwinds viral DNA for replication, has been used as a model to study ring-shaped replicative helicases. In the presence of dTTP, the gene 4 protein binds to single-stranded DNA as a hexamer and translocates unidirectionally 5′ to 3′ along the DNA strand by using the energy of dTTP hydrolysis (Egelman et al., 1995; Kim et al., 2002; Tabor and Richardson, 1981). The movement of the protein can be inhibited by a nonhydrolyzable nucleotide analog such as β,γ-methylene dTTP.

The dTTPase activity of the T7 gene 4 helicase is markedly diminished in the absence of ssDNA (Bernstein and Richardson, 1988; Patel et al., 1992; Washington et al., 1996). The mechanism of ssDNA-independent dTTP hydrolysis has been suggested to be similar to ATP hydrolysis by the F_1-ATPase (Hingorani et al., 1997; Jeong et al., 2002). It was observed by pre-steady-state experiments that only one subunit hydrolyzes dTTP at a fast rate, followed by a second one at a slower rate, a sequence that resembles the binding-change mechanism of the F_1-ATPase (Boyer, 1993). Additionally, it was reported that, in the absence of ssDNA, some subunits are noncatalytic (Hingorani et al., 1997; Jeong et al., 2002). Subsequently, a model was proposed with three catalytic subunits fixed in relation to three noncatalytic subunits alternating around the ring reflecting the α (noncatalytic) and β (catalytic) subunits of the F_1-ATPase. However, the crystal structure of the hexameric helicase domain exhibited a nucleotide binding configuration inconsistent with this model (Singleton et al., 2000). The crystal structure revealed subunits in three different states: empty, loosely bound adenosine 5′-[β, γ-imido]-triphosphate, and tightly bound adenosine 5′-[β, γ-imido]triphosphate. These different nucleotide binding conformations followed each other around the hexameric ring in repeated sequence such that subunits across the ring from each other were identical. As a result, Singleton et al. (2000) proposed a sequential pathway in which all subunits participated in dTTP hydrolysis.

The crystal structures of the helicase domain of the gene 4 protein show a catalytic core that resembles that of *Escherichia coli* RecA protein and the F_1-ATPase (Sawaya et al., 1999; Singleton et al., 2000). Amino acid sequence alignment of several proteins that hydrolyze nucleoside triphosphates shows the occurrence of a glutamate subsequent to residues of the Walker A motif (Yoshida and Amano, 1995) (Figure 1A). The crystal structure of residues 241–566 shows glutamate 343 adjacent to the γ-phosphate of the bound adenosine 5′-[β, γ-imido]triphosphate (Singleton et al., 2000) (Figure 1B). This conserved glutamate is postulated to be the catalytic base analogous to glutamate 188 of the mitochondrial F_1-ATPase (Abrahams et al., 1994), a residue thought to activate the nucleophilic water molecule for an in-line attack of the ATP γ-phosphate facilitating nucleotide hydrolysis (Story and Steitz, 1992). Although the α subunit of the F_1-ATPase is noncatalytic, it has an overall amino acid sequence similar to the β subunit. The key difference between the α and the β subunits is a glutamine in the α subunit rather than a glutamate at the position postulated to be the catalytic base in the β subunit (Matsui et al., 1996). Altering this glutamate in both *E. coli* RecA and the F_1-ATPase results in a loss of ATP hydrolysis (Campbell and Davis, 1999a, 1999b; Hu et al., 1996; Park et al., 1994; Senior and al-Shawi, 1992). Similarly, altering the analogous glutamate in the hexameric helicases *Bacillus stearothermophilus* DnaB or the RSF1010 RepA also abolishes ATP hydrolysis (Soultanas and Wigley, 2002; Ziegelin et al., 2003).

Consistent with the ssDNA binding site proposed from electron microscopy studies (Yu et al., 1996), the crystal structure of the hexameric protein shows three potential ssDNA binding loops lining the central cavity of the ring (Singleton et al., 2000). Studies of altered gene 4 proteins with mutations near loop I (residues 424–439) and loop II (residues 464–475) have further supported the involvement of these loops in ssDNA binding (Notarnicola et al., 1995; Washington et al., 1996). Loop II, analogous to the disordered mobile loop L2 of the of the *E. coli* RecA protein (Story and Steitz, 1992), contains

*Correspondence: ccr@hms.harvard.edu

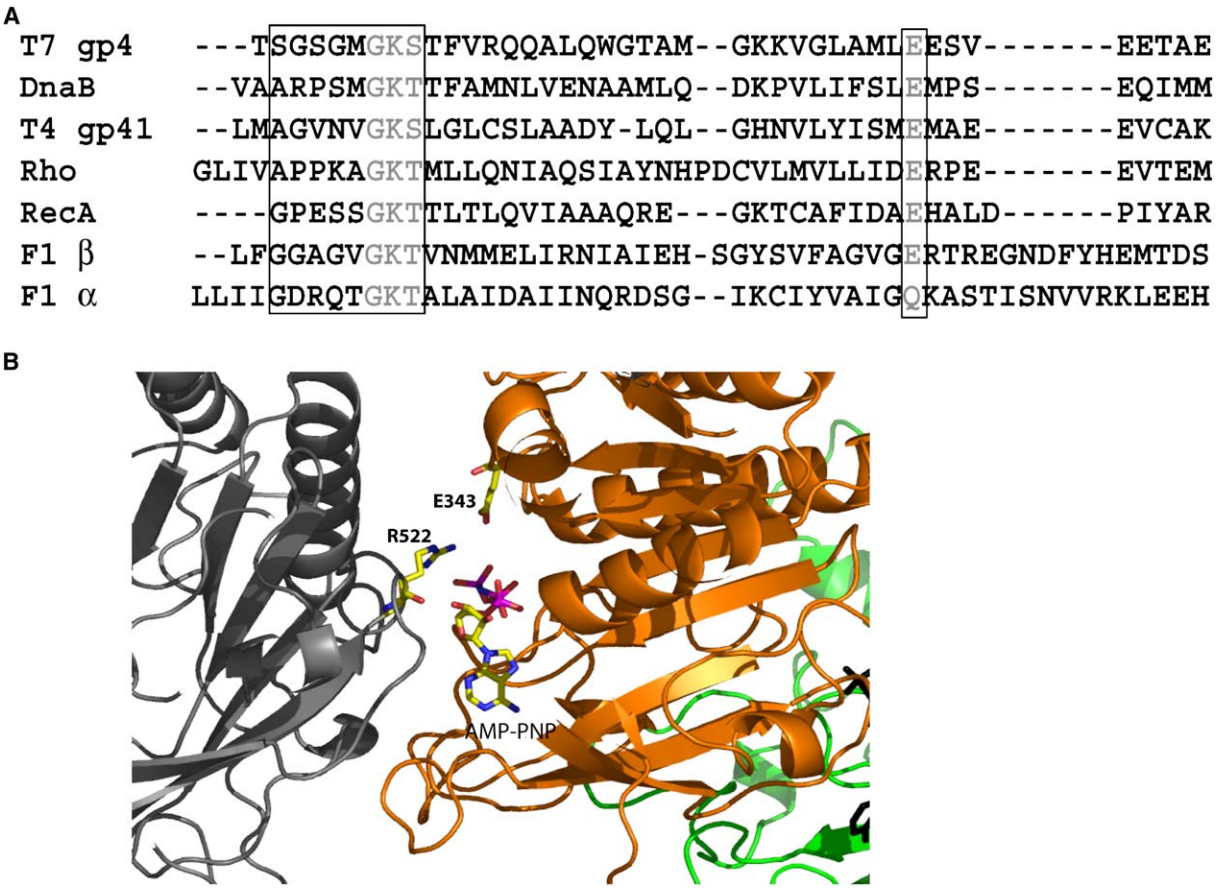

Figure 1. Catalytic Base of the T7 Gene 4 Protein

(A) Partial amino acid sequence alignment of the T7 gene 4 protein with other hexameric helicases as well as the *E. coli* transcription termination factor rho, RecA, and the F_1-ATPase β and α subunits. The highlighted residues correspond to the H1 (Walker A homology or P loop) and H1a motifs of hexameric helicases (Matson and Kaiser-Rogers, 1990).

(B) The crystal structure of T7 gene 4 helicase showing a nucleotide binding site containing 5′-[β,γ-imido]adenosine triphosphate (Singleton et al., 2000). Arginine 522 (R522) from an adjacent subunit inserts itself into the nucleotide binding site where it interacts with the γ-phosphate of the bound nucleotide acting as an arginine finger (Crampton et al., 2004). Nearby, glutamate 343 (E343) is in position to activate the nucleophilic water molecule for an in-line attack of the nucleotide γ-phosphate.

three lysine residues for potential electrostatic interactions with the negatively charged phosphate backbone of ssDNA (Figure 2).

The mechanism of dTTP hydrolysis in the T7 gene 4 protein has been thought to be fundamentally similar for both ssDNA-independent and -dependent dTTP hydrolysis pathways. Recently, it has been suggested that all six subunits participate in ssDNA-dependent dTTPase activity (Liao et al., 2005). In the presence of ssDNA, it was observed that up to four subunits in the hexamer hydrolyze dTTP at a fast rate. This arrangement more closely resembles the crystal structure, although the protein in the crystal structure did not have ssDNA bound (Singleton et al., 2000). Liao et al. (2005) reason that four subunits capable of hydrolyzing dTTP eliminate the F_1-ATPase-derived model for ssDNA-dependent hydrolysis and further suggest that mechanochemical considerations point toward a six-catalytic-subunit model. Additionally, the authors performed inhibition experiments using a DNA binding-deficient mutant that suggested a sequential mechanism for the coupling of dTTP hydrolysis and ssDNA binding.

In the present study, we show that glutamate-343 of the gene 4 protein is essential for the hydrolysis of dTTP and, consequently, for translocation and unwinding activity. Additionally, we show that, in the absence of positive charges on loop II, the altered gene 4 protein gp4-K_{loopII}A lacks the ability to bind ssDNA. Consequently, we exploit the noncatalytic nature of gp4-E343 and gp4-K_{loopII}A to investigate the mechanism of gene 4 protein-catalyzed dTTP hydrolysis. Using mixed oligomers of wild-type and altered monomers, we demonstrate that all subunits are catalytic and that the binding of single-stranded DNA is necessary for sequential hydrolysis as subunits hydrolyze dTTP independently during ssDNA-independent dTTP hydrolysis.

Results

Construction of Noncatalytic Subunits

To abolish the ability of the gene 4 protein to hydrolyze dTTP, we mutated both the residue postulated to be the catalytic base for the dTTPase reaction and the region thought to be an important contributor to ssDNA

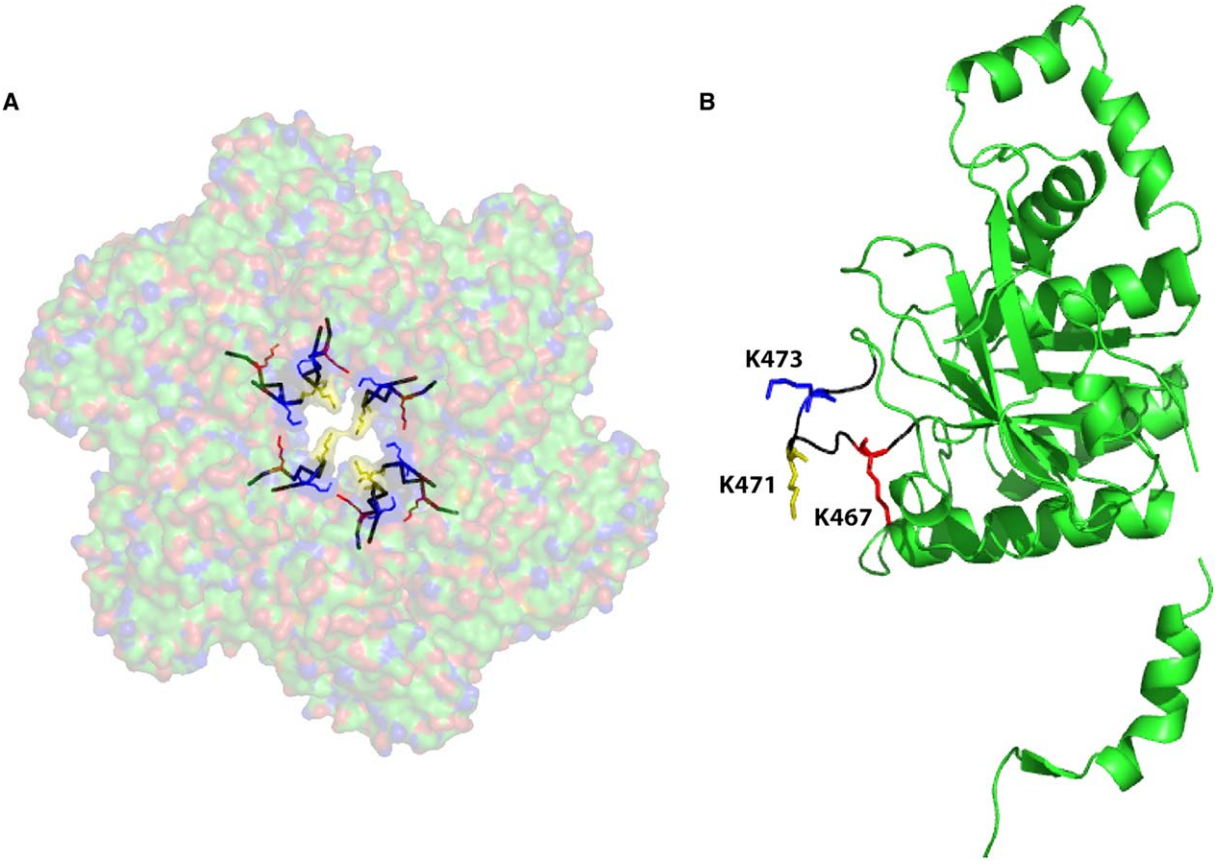

Figure 2. DNA Binding Loop II of the T7 Gene 4 Protein

(A) The crystal structure of T7 gene 4 helicase showing loop II, including lysine 467 (red), lysine 471 (yellow), and lysine 473 (blue), is located within the central cavity of the ring (Singleton et al., 2000).

(B) Side view of an individual subunit displaying the arrangement of the lysine residues on loop II.

binding. Amino acid sequence alignment, along with crystallographic data, identifies glutamate 343 as the most likely candidate for the catalytic base of the T7 gene 4 helicase (Figure 1). To explore this possibility, glutamate 343 was replaced with a glutamine residue to generate gp4-E343Q, a subunit whose nucleotide binding site resembles that of the noncatalytic α subunit of the F_1-ATPase (Matsui et al., 1996). Thus, without the carboxylate to activate the nucleophilic water molecule, dTTP hydrolysis should not occur in the gene 4 protein. The loop II region of the gene 4 protein extends into the central cavity recognized as the location of ssDNA binding (Figure 2). To eliminate the ability of the gene 4 protein to bind ssDNA, the lysine residues of loop II at positions 467, 471, and 473 were substituted with alanines to remove potential electrostatic interactions with the negatively charged phosphate backbone of bound ssDNA. In the absence of these lysine residues, dTTP hydrolysis activity dependent on binding ssDNA should be hindered. The ability of the mutated genes to complement the function of gene 4 in vivo was examined in a phage complementation assay. T7Δ4–1, lacking the gene 4, can grow only in *E. coli* cells harboring a plasmid with a functional gene 4 (Mendelman et al., 1992). Plasmids harboring gp4-E343Q or gp4-K_{loopII}A were unable to support the growth of the T7Δ4–1 phage (data not shown). Thus, these alterations of glutamate 343 and loop II disturbed essential functions of the gene 4 protein.

Protein Purification and Oligomerization

In order to analyze the role of glutamate 343 and loop II biochemically, we purified the genetically altered gene 4 proteins, gp4-E343Q and gp4-K_{loopII}A, from cells harboring the cloned genes. The overall yield of the altered proteins was greater than for wild-type gene 4 protein. Both gp4-E343Q and gp4-K_{loopII}A comprised approximately 30% of the total protein in the lysate as compared to less than 1% for the wild-type. The low expression of wild-type protein is due to its toxicity, presumably as a result of dTTP hydrolysis. The altered proteins behaved similarly during the purification, and the purity was greater than 95% as judged by Coomassie staining of proteins after SDS gel electrophoresis. The genetically altered proteins form oligomers as well as does wild-type gene 4 protein (see Figure S1 in the Supplemental Data available with this article online).

Glutamine at Position 343 Abolishes dTTP Hydrolysis and DNA Unwinding

The inability of the genetically altered helicase to support T7 phage growth suggests that glutamate 343 plays an essential role in the helicase mechanism. Given that

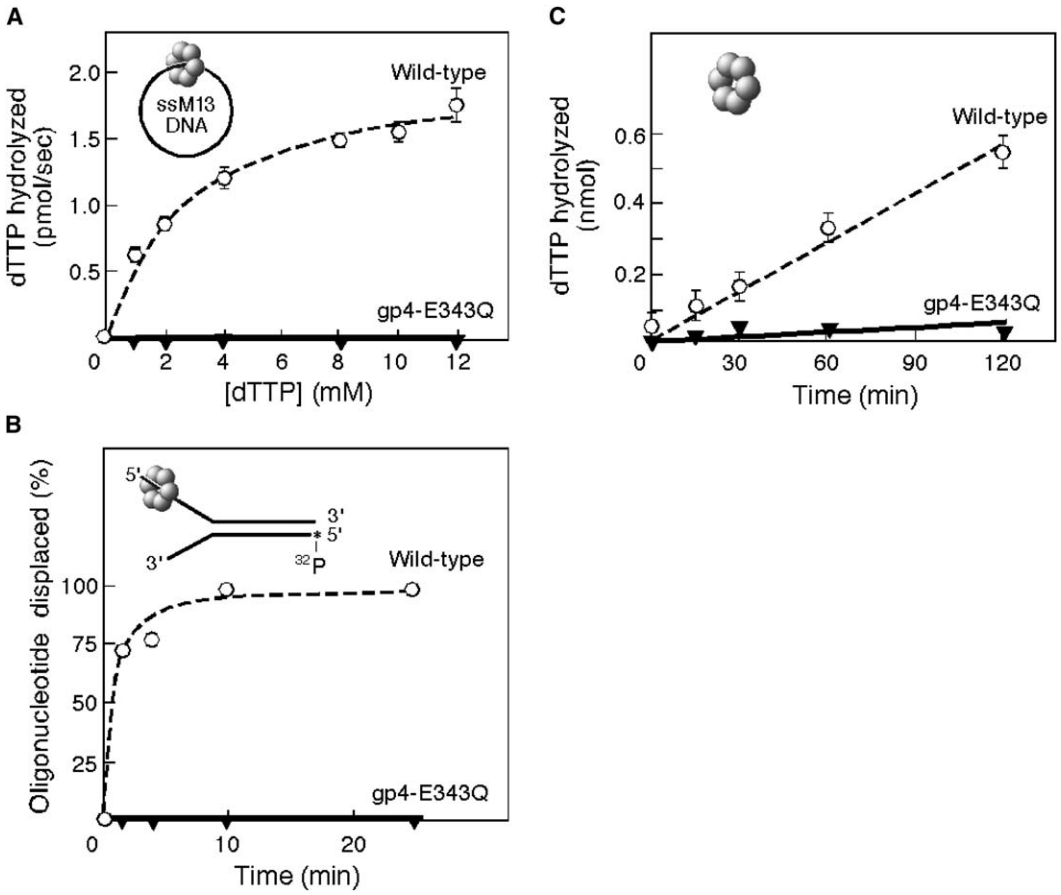

Figure 3. Helicase-Associated Activities of Wild-Type Gene 4 Protein and gp4-E343Q

The error bars represent the standard deviation of three or more independent experiments.

(A) Single-stranded DNA-dependent dTTP hydrolysis catalyzed by gene 4 protein (○) and gp4-E343Q (▼) (inset). Gene 4 protein catalyzes the ssDNA-dependent hydrolysis of dTTP to dTDP and Pi. Each reaction contained 1.1 nM M13 ssDNA, the indicated concentration of dTTP (0.5, 1, 2, 4, 6, and 8 mM), 0.1 μCi of [α-^{32}P] dTTP, and 120 nM of wild-type gene 4 protein or gp4-E343Q (this and all concentrations are given as monomers of gene 4 protein). At each dTTP concentration, the reactions were incubated at 37°C for 30 min, and then the product of the reaction was analyzed by TLC as described in Experimental Procedures. The rate of dTTP hydrolysis is plotted against the initial concentration of dTTP.

(B) DNA unwinding by wild-type (○) and gp4-E343Q proteins (▼). The helicase substrate consists of a 95 base oligonucleotide with 55 bases of a 75 base oligonucleotide annealed to its 3′ end as diagrammed in the inset. The 75-mer was 5′- ^{32}P-labeled so that its position in a polyacrylamide gel can be detected and the amount released measured. The reactions contained 1 nM helicase substrate, 5 mM dTTP, and 100 nM gene 4 protein. The reactions were allowed to progress for the indicated times (0, 2, 4, 10, and 25 min) at 37°C followed by quenching with EDTA. The products were separated by electrophoresis using a 10% TBE gel. The change in the amount of labeled 75-mer separated from the partial duplex DNA substrate was calculated and expressed as percent of total helicase substrate.

(C) Single-stranded DNA-independent hydrolysis of dTTP by wild-type (○) and gp4-E343Q (▼) (inset). Each reaction contained 10 mM dTTP, 1 μCi of [α-^{32}P] dTTP, and 120 nM gene 4 protein. The reactions were incubated at 37°C for the indicated times (0, 15, 30, 60, and 120 min) followed by quenching with EDTA. The product of the reaction was analyzed by TLC as described in Experimental Procedures. The amount of dTTP hydrolyzed is plotted against the incubation time.

the altered protein was able to form oligomers similar to wild-type gene 4 protein, we measured the ability of gp4-E343Q to hydrolyze dTTP in the presence of ssDNA. The crystal structure of the helicase domain (Figure 1B) places glutamate 343 in position to activate the nucleophilic water molecule for an in-line attack of the dTTP γ-phosphate (Singleton et al., 2000). gp4-E343Q has no discernible ssDNA-dependent dTTPase activity compared to wild-type gene 4 protein (Figure 3A). Since the ability of the gene 4 protein to unwind DNA is dependent upon the energy derived from the hydrolysis of dTTP, it is not surprising that the loss of glutamate 343 leads to a loss of helicase activity (Figure 3B).

Gene 4 protein hydrolyzes dTTP at a much slower rate without single-stranded DNA (Bernstein and Richardson, 1988; Patel et al., 1992; Washington et al., 1996). Removal of the carboxylate responsible for facilitating dTTP hydrolysis should also destroy the ability of the enzyme to hydrolyze dTTP in the absence of ssDNA. As shown in Figure 3C, gp4-E343Q has little ssDNA-independent dTTP hydrolysis activity.

gp4-E343Q Binds ssDNA with High Affinity in the Presence of dTTP

The binding of gene 4 protein to ssDNA is dTTP dependent (Matson and Richardson, 1985). Gene 4 helicase-ssDNA binding studies frequently make use of the non-hydrolyzable analog β,γ-methylene dTTP to inhibit translocation and to allow tight binding of ssDNA (Hingorani and Patel, 1993; Matson and Richardson, 1985)

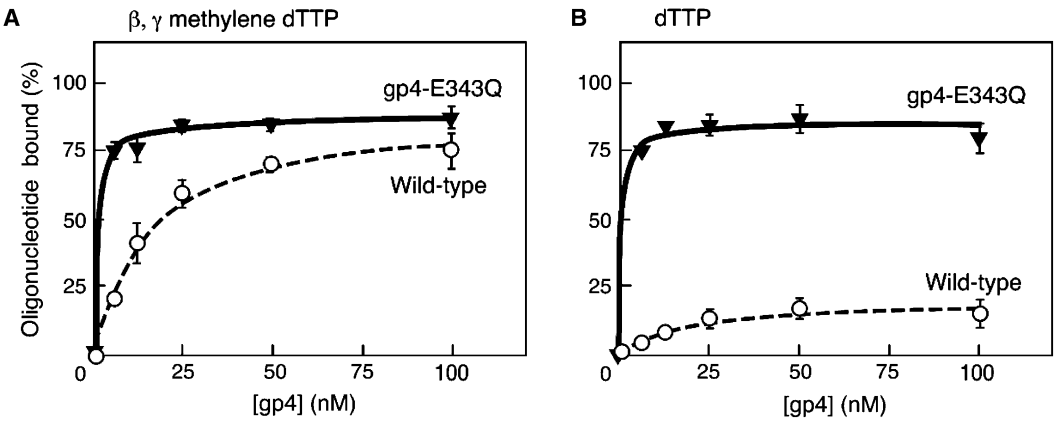

Figure 4. Single-Stranded DNA Binding Affinity of Gene 4 Protein Measured by Nitrocellulose Filter Binding

Each reaction contained 1 nM 5′-^{32}P-labeled 50-mer oligonucleotide, the indicated amount of either wild-type gene 4 protein or gp4-E343Q (0–0.1 μM), and either 5 mM β,γ-methylene (A) or 5 mM dTTP (B) for 30 min incubation at 37°C. Reactions were filtered through a nitrocellulose membrane, and the amount of radioactivity bound to the nitrocellulose was measured and expressed as a percent of total oligonucleotide added. Wild-type (○), gp4-E343Q (▼). The error bars represent the standard deviation of four independent experiments.

(Figure 4). The K_d of wild-type for oligonucleotide is 2-fold less in the presence of β,γ-methylene dTTP (7 nM) (Figure 4A) than dTTP (14 nM) (Figure 4B). gp4-E343Q binds oligonucleotide in the presence of dTTP with a 10-fold higher efficiency than that observed for wild-type gene 4 protein in presence of dTTP (Figure 4B). In the absence of the catalytic base, the gene 4 protein has little dTTPase activity in the presence of absence of DNA, suggesting that the altered subunits are noncatalytic (Figure 3). The noncatalytic character of gp4-E343Q is further revealed by the tight binding of gp4-E343Q to ssDNA in the presence of dTTP that is only observed in the wild-type protein when in the presence of the nonhydrolyzable analog β,γ-methylene dTTP (Figure 4B). Using gp4-E343Q, it is possible to test previous reports that some subunits of the oligomeric ring are noncatalytic (Hingorani et al., 1997; Jeong et al., 2002).

dTTP Hydrolysis by Heterooligomers Composed of Wild-Type and gp4-E343Q Subunits

The identical subunits of the active gene 4 protein oligomer are all apparently capable of binding and hydrolyzing dTTP. Biochemical studies have led to the postulation that, in the absence of ssDNA, three catalytic sites are fixed in relation to three noncatalytic sites alternating around the hexameric ring (Hingorani et al., 1997; Jeong et al., 2002). Conversely, crystallographic data suggest a sequential mechanism with each subunit contributing to dTTP hydrolysis (Singleton et al., 2000). In the presence of ssDNA, it has been postulated that all subunits are involved in gene 4 protein-catalyzed ssDNA-dependent dTTP hydrolysis (Liao et al., 2005). The fact that gp4-E343Q binds ssDNA tightly without dTTP hydrolysis provides a unique opportunity to examine the effect of a noncatalytic subunit on the dTTP hydrolysis mechanism of the gene 4 protein. In this experiment, gp4-E343Q and wild-type gene 4 protein are mixed at various concentrations to yield oligomers containing different ratios of the two gene 4 proteins. A previous study has conclusively shown random mixing of subunits at the monomer level (Lee and Richardson, 2002), and therefore a Poisson distribution of subunits in the mixed oligomers is expected.

Heterooligomers in which one or more of the subunits is deficient in dTTP hydrolysis have, in earlier studies, exhibited significantly decreased ssDNA-dependent dTTPase activity, leading to the conclusion that the subunits in the hexamer work in a cooperative manner (Crampton et al., 2004; Notarnicola and Richardson, 1993; Patel et al., 1994). Cooperativity implies that dTTP hydrolysis on any catalytically active subunit is dependent in some way upon the other subunits of the oligomeric ring. However, these prior studies have used altered subunits that disrupted either nucleotide binding, DNA binding, or intersubunit communication (Crampton et al., 2004; Notarnicola and Richardson, 1993; Patel et al., 1994). The modification of glutamate 343 to glutamine leads to a noncatalytic subunit in which all the necessary residues for catalysis of bound dTTP are functional except the catalytic base.

If an active gene 4 protein oligomer contains some number of subunits that are noncatalytic, then the inclusion of a small percentage of gp4-E343Q subunits should have a minimal affect on dTTP hydrolysis proportional to the number of noncatalytic subunits. Additionally, if dTTP hydrolysis is carried out by subunits randomly, then the probability of a wild-type or gp4-E343Q subunit hydrolyzing dTTP depends on the ratio of the two subunits. As shown in Figure 5A, only a fraction of the total protein mixture needs to be gp4-E343Q to nearly abolish ssDNA-dependent dTTPase activity. This result strongly suggests that there are no noncatalytic subunits and that all six subunits must be catalytically active for ssDNA-dependent dTTP hydrolysis. Furthermore, a sequential mechanism of dTTP hydrolysis around the hexameric ring is implied, given that the ssDNA-dependent dTTP hydrolysis cannot persist when approximately one gp4-E343Q subunit is in a ring to disturb the sequence.

Conversely, during ssDNA-independent dTTPase, there is a proportional loss of dTTPase activity corresponding to the percentages of the catalytic wild-type subunits and noncatalytic gp-E343Q subunits (Figure 5B). The linear decrease in ssDNA-independent

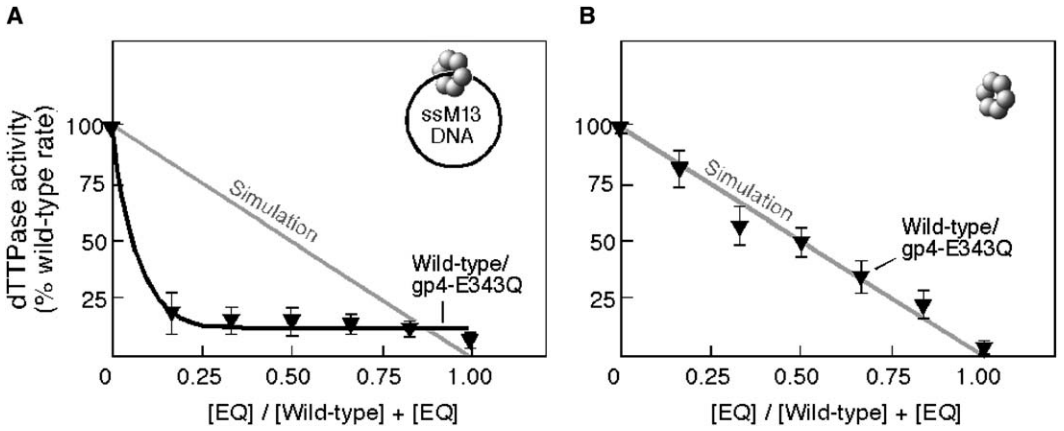

Figure 5. dTTPase Activity of Heterohexamers Composed of Wild-Type Gene 4 Protein and gp4-E343Q

(A) Each reaction contained 1.1 nM M13 ssDNA, 5 mM dTTP, 0.1 μCi of [α-^{32}P] dTTP, and 120 nM indicated mixture of gene 4 proteins. (B) Each reaction contained 10 mM dTTP, 1 μCi of [α-^{32}P] dTTP, and 120 nM of the indicated mixture of gene 4 proteins. Before the reaction, wild-type gene 4 protein was mixed with gp4-E343Q at the indicated percentage of subunits in the absence of dTTP. At each percentage, the reactions were incubated at 37°C for 30 min (A) or 120 min (B) and then quenched by EDTA. The product of the reaction was analyzed by TLC as described in Experimental Procedures. The dTTPase activity in the presence (A) or absence (B) of single-stranded DNA is expressed as percentage of dTTP hydrolyzed with respect to the value obtained for wild-type gene 4 homohexamer, which was 1.4 pmol/s and 0.08 pmol/s, respectively. In both panels, the gray line represents a simulation of a random mechanism of dTTP hydrolysis. The error bars represent the standard deviation of four independent experiments.

dTTP hydrolysis suggests that, in the absence of ssDNA, subunits of the oligomer hydrolyze dTTP independently. Since the arrangement of gp4-E343Q and wild-type subunits in an oligomer is random, ssDNA-independent hydrolysis of dTTP cannot be cooperative. This result suggests that ssDNA stimulates dTTPase activity through the organization of independent subunits randomly hydrolyzing dTTP into subunits acting together to hydrolyze dTTP.

Interestingly, although ssDNA-dependent dTTPase is abolished by inclusion of gp4-E343Q into hexamers (Figure 5A), the remaining wild-type subunits are able to hydrolyze dTTP at a very slow rate independently of the other subunits. In a manner similar to ssDNA-independent dTTP hydrolysis, the dTTPase activity of hexamers with tightly bound ssDNA linearly decreases in response to each additional gp4-E343Q subunit (Figure S2). Thus, the striking decrease in the ssDNA-dependent dTTPase activity by mixed oligomers could be due to persistent binding of ssDNA binding to the gp4-E343Q subunit (Figure 4B). To further test whether there is a sequential mechanism of dTTP hydrolysis around the hexameric ring, we repeated the mixing studies with an altered gene 4 protein that lacks the ability to bind ssDNA.

dTTP Hydrolysis by Heterooligomers Composed of Wild-Type and gp4-K$_{loopII}$A Subunits

The crystal structure of the hexameric protein shows residues 464–475 forming a loop, referred to as loop II, located in the central cavity of the ring (Singleton et al., 2000). Loop II occupies a place analogous to the mobile disordered L2 loop of the RecA protein (Story and Steitz, 1992) and contains three lysine residues for potential electrostatic interactions with the negatively charged phosphate backbone of bound ssDNA (Figure 2). The substitution of alanines for lysine 467, lysine 471, and lysine 473 results in the altered subunits having a dramatic loss of affinity for ssDNA (Figure 6A). This result supports the postulation that loop II is involved in binding of ssDNA (Singleton et al., 2000). In a manner similar to gp4-E343Q, Figure 6B shows that only a small percentage of the total protein mixture needs to be gp4-K$_{loopII}$A in order to abolish ssDNA-dependent dTTPase activity (Figure 6B). This is the expected outcome if DNA binding and dTTPase activity are coupled in a sequential pathway around the ring. Substantiating the postulated role of loop II in ssDNA binding, mixtures of gp4-K$_{loopII}$A and wild-type monomers in the absence of ssDNA do not result in a decrease of dTTP hydrolysis (Figure S3).

Discussion

Glutamate 343 Functions as a Catalytic Base

Structural and biochemical studies of the gene 4 helicase suggest a mechanism of energy transduction in which highly conserved residues comprise an engine that couples the energy derived from dTTP hydrolysis to the translocation of the protein along ssDNA. The conserved glutamate at position 343, adjacent to the γ-phosphate of the bound adenosine 5′-[β, γ-imido]triphosphates in the crystal structure (Singleton et al., 2000), is thought to act as a catalytic base to extract a proton from the water molecule that subsequently engages in a nucleophilic attack of dTTP. The loss of ATP hydrolysis when the analogous glutamate is altered in the helicases *B. stearothermophilus* DnaB and RSF1010 RepA supports this assumption (Soultanas and Wigley, 2002; Ziegelin et al., 2003).

In the present study, we show that replacing glutamate 343 of the T7 gene 4 protein (gp4-E343Q) results in a protein that is unable to hydrolyze dTTP (Figure 3). The catalytic base should be equally important for both ssDNA-dependent and -independent hydrolysis. Accordingly, gp4-E343Q has no measurable dTTPase under either condition. However, gp4-E343Q binds ssDNA with high

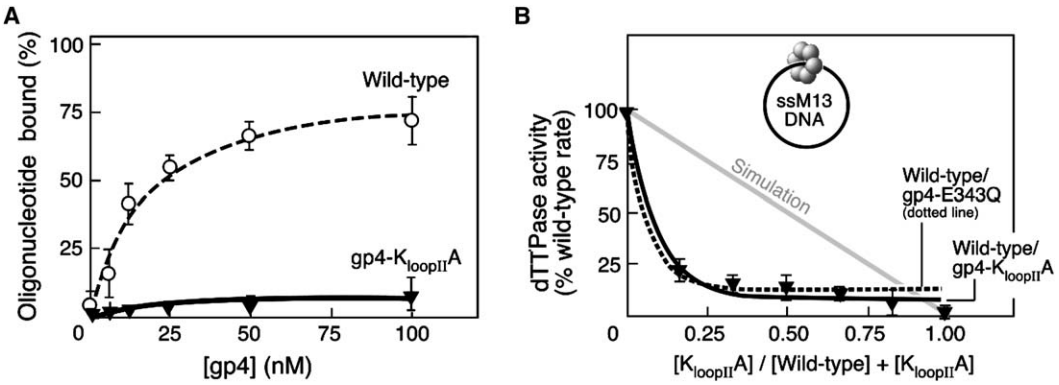

Figure 6. dTTPase Activity of Heterohexamers Composed of Wild-Type Gene 4 Protein and Single-Stranded DNA Binding-Deficient gp4-K$_{loopII}$A

(A) Single-stranded DNA binding affinity of gp4-K$_{loopII}$A measured by nitrocellulose filter binding. Each reaction contained 1 nM 5′-^{32}P-labeled 50-mer oligonucleotide, the indicated amount of either wild-type gene 4 protein (○) or gp4- gp4-K$_{loopII}$A (▼) (0–0.1 μM), and 5 mM β,γ-methylene dTTP for 30 min incubation at 37°C. Reactions were filtered through a nitrocellulose membrane, and the amount of radioactivity bound to the nitrocellulose was measured and expressed as a percent of total oligonucleotide added.

(B) dTTPase activity of heterohexamers composed of wild-type gene 4 protein and gp4-K$_{loopII}$A in the presence of ssDNA. Each reaction contained 1.1 nM M13 ssDNA, 5 mM dTTP, 0.1 μCi of [α-^{32}P] dTTP, and 120 nM indicated mixture of gene 4 proteins. Before the reaction, wild-type gene 4 protein was mixed with gp4-K$_{loopII}$A at the indicated percentage of subunits in the absence of dTTP. At each percentage, the reactions were incubated at 37°C for 30 min and then quenched by EDTA. The product of the reaction was analyzed by TLC as described in Experimental Procedures. The dTTPase activity is expressed as percentage of dTTP hydrolyzed with respect to the value obtained for wild-type gene 4 homohexamer, which was 1.4 pmol/s. The gray line shows a simulation of a random mechanism of dTTP hydrolysis, while the broken line represents the results obtained with heterohexamers of wild-type and gp4-E343Q under the same conditions. The error bars represent the standard deviation of three or more independent experiments.

affinity (Figure 4). In the absence of the catalytic base, dTTP bound to gp4-E343Q has the characteristics of a nonhydrolyzable analog, and, as a consequence, gp4-E343Q resembles a noncatalytic subunit.

All Subunits Are Catalytic

In the absence of ssDNA, the gene 4 protein hydrolyzes dTTP at a very low rate (Bernstein and Richardson, 1988; Patel et al., 1992). We observe that single-stranded DNA-independent dTTP hydrolysis activity decreases linearly with the serial addition of noncatalytic subunits (Figure 5B). Thus, in the absence of ssDNA, all subunits are catalytic and hydrolyze dTTP independently from the other subunits. It has been reported that, during the pre-steady state in the absence of ssDNA, the release of product from one site triggers the hydrolysis of dTTP bound to another (Jeong et al., 2002). Hence, ssDNA-independent dTTP hydrolysis has been postulated to be by a cooperative mechanism. However, our data show that ssDNA-independent dTTPase activity by individual subunits is unaffected by the nature of the other subunits. It is possible that the pre-steady state of ssDNA-independent dTTP hydrolysis is not representative of subsequent turnovers, and that, without ssDNA, only the first turnover of dTTP hydrolysis resembles ssDNA-dependent dTTP hydrolysis. In this scenario, the absence of ssDNA causes sequential dTTP hydrolysis to break down after the first two subunits cooperatively hydrolyze dTTP, leaving the subunits to hydrolyze dTTP independently at a slow rate.

In the presence of ssDNA, Liao et al. (2005) observe that up to four subunits in the hexamer hydrolyze dTTP at a fast rate. However, they suggest that mechanochemical factors indicate a six-catalytic-subunit mechanism of ssDNA-dependent dTTP hydrolysis. We show that a small amount of the noncatlatytic subunit gp4-E343Q in an oligomer otherwise composed of wild-type subunits leads to the loss of nearly all ssDNA-dependent dTTP hydrolysis activity (Figure 5A). Thus, our data confirm that an active hexamer contains six subunits capable of hydrolyzing dTTP and negate the F_1-ATPase-derived model, which includes noncatalytic subunits.

The Lysines on Loop II Are Essential for ssDNA Binding

Replacing lysines 467, 471, and 473 with alanine in the T7 gene 4 protein (gp4-K$_{loopII}$A) results in a protein that is unable to bind ssDNA (Figure 6A). This result suggests that the electrostatic interactions with the negatively charged phosphate backbone of bound ssDNA supplied by loop II are a major component of ssDNA binding. The crystal structure of the hexameric helicase region with bound nucleotides exhibited three subunit conformations: low occupancy with adenosine 5′-[β, γ-imido]-triphosphates, high occupancy with adenosine 5′-[β, γ-imido]triphosphates, and no bound nucleotide (Singleton et al., 2000). The subunit conformations associated with the empty and low occupancy sites exhibit a displacement of loop II by 6–7 Å. This distance correlates well with the measured rate of translocation along ssDNA of two to three nucleotides per dTTP reported by Kim et al. (2002).

Sequential Model of Single-Stranded DNA-Dependent dTTP Hydrolysis

The crystal structure of the hexameric helicase region showed nucleotides bound in a manner consistent with sequential hydrolsysis of dTTP around the hexameric ring (Singleton et al., 2000). The three observed conformations of low occupancy, high occupancy, and empty followed each other around the hexameric ring with identical subunits located directly across from each

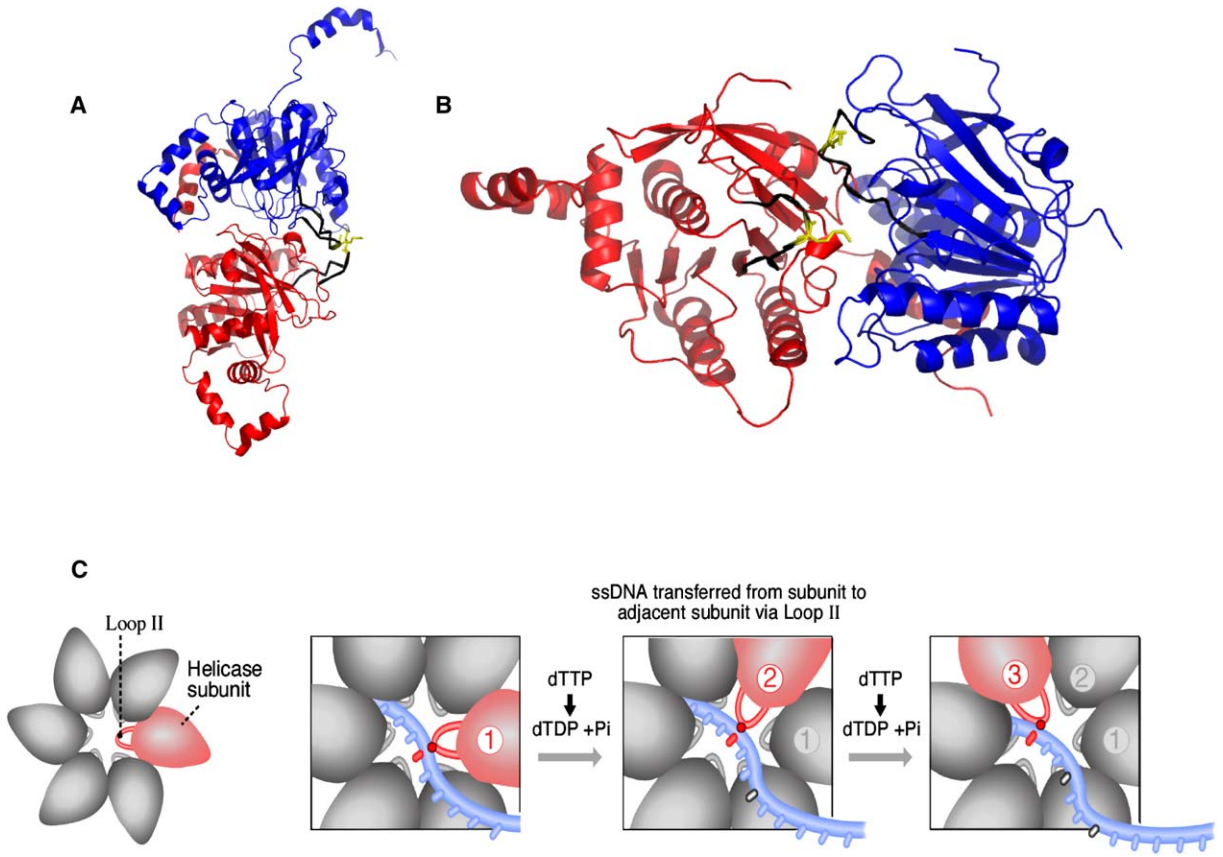

Figure 7. Sequential Model of ssDNA-Dependent dTTP Hydrolysis by the T7 Gene 4 Helicase

The configurations of loop II from the empty (blue) and loosely bound 5′-[β,γ-imido]adenosine triphosphate (red) found in the crystal structure in the absence of ssDNA are shown down from the N terminus (A) and out from the center of the ring (B) (Singleton et al., 2000). The loops II of these subunits are positioned very close to each other, with the lysines at position 471 overlapping each other in the vertical plane of the central cavity. This capacity of the loops II from adjacent subunits to be in this conformation suggests a mechanism for sequential DNA transfer from subunit to adjacent subunit. (C) The proposed mechanism in which the DNA strand stimulates the dTTPase activity of the subunit to which it is bound (red, 1). Following the hydrolysis of dTTP, the DNA strand is transferred to an adjacent subunit (red, 2), with dTTP bound to continue the process in a sequential manner around the ring.

other within the ring. However, the crystallized protein does not have ssDNA bound in its central cavity, and our results suggest that the mechanisms of ssDNA-dependent and -independent dTTP hydrolysis are very different.

Additionally, we show that a small percentage of gp4-$K_{loopII}A$ in an oligomer otherwise composed of wild-type subunits leads to the loss of all ssDNA-dependent dTTP hydrolysis activity (Figure 6B). This result suggests that ssDNA is passed between adjacent subunits to perpetuate sequential dTTP hydrolysis around the ring. In the hexameric crystal structure, the lysine residues at position 471 on the loops II of adjacent empty and low occupancy subunits are orientated such that these residues are stacked upon each other in the vertical plane (Figures 7A and 7B).

Our results suggest that the binding of ssDNA into the central channel of the gene 4 protein ring induces a transformation of catalytically independent subunits into a motor of unified subunits working in cooperative manner to rapidly hydrolyze dTTP. Consequently, we propose a model in which dTTP hydrolysis occurs only at subunits where ssDNA is bound and that the release of dTDP and Pi allows the DNA strand to be passed from the now-empty subunit to an adjacent subunit with dTTP bound (Figure 7C). This model is complementary to the models of sequential dTTP hydrolysis and sequential powerstrokes for DNA translocation proposed by Singleton et al. (2000) and Liao et al. (2005), respectively. Presumably, conformational changes induced by the binding of ssDNA are responsible for the stimulation dTTP hydrolysis (Ahnert et al., 2000; Picha et al., 2000; Yong and Romano, 1995). In the absence of ssDNA, the rate-limiting step of dTTP hydrolysis is the release of dTDP (Jeong et al., 2002). Without ssDNA, dTTP is likely bound suitably within the catalytic sites for hydrolysis, but one or more catalytically important residues are not properly placed for the efficient release of dTDP. The observations that P_i release is the rate-limiting step in ssDNA-dependent dTTP hydrolysis (Liao et al., 2005) and that single-stranded DNA binds to one to two subunits in the hexamer at one time (Yu et al., 1996) suggest a straightforward mechanism for the stimulation of dTTPase activity through the bound DNA strand facilitating the release of dTDP from the subunit to which it is bound.

Recently, a similar study of mixing catalytically inactive with active subunits was done on ClpX, a member of the hexameric ring-shaped ATPases of the AAA+ superfamily (Martin et al., 2005). Martin et al. observed that the mechanistic pathway of ATP hydrolysis by ClpX did not follow a strict sequential progession. The difference in the mechanisms of nucleotide hydrolysis by T7 helicase and ClpX may represent subtle divergence of RecA-like and AAA+ proteins.

Experimental Procedures

Materials

Oligonucleotides were obtained from the Bioplomer Laboratory at Harvard Medical School and Integrated DNA Technologies. T4 polynucleotide kinase was purchased from New England Biolabs. QuickChange II Site-Directed Mutagenesis Kit was purchased from Stratagene. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. dTTP, dTDP, and β,γ-methylene dTTP were purchased from USB Corp. Polyethyleneimine cellulose thin-layer chromatography (TLC) plates were from J.T. Baker. *E. coli* strain DH5α was from Invitrogen, and HMS174(DE3) was from Novagen.

Construction of Plasmids

pET11gp4-63 (Takahiro Kusakabe, Harvard Medical School) was constructed by inserting the gene 4-coding DNA fragment into pET11b (Novagen) between HindIII and NdeI sites. In this gene 4-coding plasmid, the internal start codon at position 64 was replaced with the codon for glycine to avoid coproduction of the 56 kDa protein (Mendelman et al., 1992). Mutations were introduced using the Quickchange II Mutagenesis kit (Stratagene) and the following primers: E343Q(+) 5′-GGCTTAGCGATGCTTCAGTCCGTTGA GGA GACC-3′, E343Q(−) 5′-GGTCTCCTCAACGGACTCCTGAAGC ATCGCTA AGCC-3′, K473A(+) 5′-CTTAAGAACCCAGACAAAGG TGCGGCACATGAGGAAGGTGCGCCC-3′, K473A(-) 5′-GGGCGCA CCTTCCTCATGTGCCGCACCTTTGTCTGGGTTCTT AAG-3′, K467/471/473A(+) 5′-GTCGTAATTTGTCACCTTGCGAACCCAGAC GCA GGT GCG-3′, K467/471/473A(-) 5′-CGCACCTGCGTCTGGGTTCGC AAGG TGACAAATTACGAC-3′. The mutated gene 4-coding regions (underlined sequence in oligonucleotide) were confirmed by DNA sequence analysis.

Protein Overproduction and Purification

Gene 4 proteins were purified by polyethylene glycol (Fluka, PEG_{4000}) precipitation of cell lysate followed by chromatography of the resuspended pellet on phosphocellulose and ATP-affinity columns as described previously (Lee and Richardson, 2001).

Oligomerization of Gene 4 Protein

The ability of gene 4 proteins to form hexamers was determined by analyzing the protein on a nondenaturing 10% polyacrylamide gel with a running buffer of 20 mM Tris-HCl (pH 7.5), 190 mM glycine, and 10mM $MgCl_2$. Each reaction contained 0.8 μM protein, 5 mM β,γ-methylene dTTP, 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 50 mM potassium glutamate. After staining with Coomassie blue, oligomerization of gene 4 proteins was verified by protein bands of greater than 300 kDa (Notarnicola et al., 1995).

dTTPase Assay for Gene 4 Protein

Gene 4 protein catalyzes the ssDNA-dependent hydrolysis of dTTP, a reaction coupled to its translocation on ssDNA (Matson and Richardson, 1983). In the absence of single-stranded DNA, the gene 4 protein hydrolyzes dTTP at very low rate (Bernstein and Richardson, 1988; Patel et al., 1992). The reaction (10 μL) contained dTTP at the concentration indicated in the text (5 or 10 mM) as well as either 0.1 μCi or 1 μCi of [α-^{32}P]dTTP, 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 50 mM potassium glutamate, and the indicated amount of gene 4 protein either in the presence or absence of 1.1 nM M13 ssDNA. After incubation at 37°C for 0, 5, 10, or 20 min in the presence of ssDNA or 0, 15, 30, 60, and 120 min without ssDNA, the reactions were terminated by adding EDTA to a final concentration of 250 mM. The reaction mixture was spotted onto a polyethyleneimine cellulose TLC plate and developed with a solution containing 1 M formic acid and 0.8 M LiCl to separate nucleoside triphosphates from nucleoside diphosphates. The amount of [α-^{32}P]dTDP formed was determined using a Fuji BAS 1000 Bioimaging Analyzer.

DNA Binding Assay

DNA binding affinity of gene 4 protein was measured by nitrocellulose filter binding. The reaction (10 μL) containing 1 nM 5′-^{32}P-labeled 50-mer oligonucleotide (5′-^{32}P -ATGACCATGATTTCGACGTTTTTTT TTTTGGGGAT CCTCTAACCTGCGCA-3′), 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 50 mM potassium glutamate was incubated with the indicated amounts of gene 4 protein in the presence of 5 mM dTTP or β,γ-methylene dTTP for 30 min at 37°C. The reaction mixture was loaded onto two layers of filters: a nitrocellulose membrane (0.45 μm, Bio-Rad) laid atop a Zeta-Probe membrane (Bio-Rad) fixed on a Dot microfiltration apparatus (Bio-Rad). The protein-DNA complex bound to the nitrocellulose membrane, and the free DNA on the Zeta-Probe membrane were determined by measuring the radioactivity in each of the two membranes.

DNA Unwinding Assay for Gene 4 Protein

A direct assay of helicase activity measures the release of a radioactively labeled oligonucleotide partially annealed to a complementary ssDNA. A helicase substrate was prepared by annealing a 5′-phosphoryl-75-mer oligonucleotide (5′-P-CGCCGGGTACCGAGCT CGAATTCACTGGCCGTCGTTTTACAACGTCGTG ACATGCCT$_{19}$-3′) to a 5′-^{32}P-labeled 95-mer oligonucleotide (5′-^{32}P -T$_{39}$GGCATG TCAC GACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTAC CCGGC G-3′) in 30 mM HEPES (pH 7.5), and 100 mM potassium acetate. The reactions (10 μL) contained 1 nM DNA substrate, 5 mM dTTP, 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 50 mM potassium glutamate, and 100 nM gene 4 protein. After incubation for 0, 1.5, 4, 10, and 25 min at 37°C, the reactions were terminated by the addition of EDTA to a final concentration of 250 mM and then loaded onto a 10% nondenaturing polyacrylamide gel. The amount of radioactively labeled oligonucleotides separated from the partial duplex substrate by the gene 4 protein was determined using a Fuji BAS 1000 Bioimaging Analyzer.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/21/2/165/DC1/.

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