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Oligomeric States of Bacteriophage T7 Gene 4 Primase/Helicase

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Electron microscopic and crystallographic data have shown that the gene 4 primase/helicase encoded by bacteriophage T7 can form both hexamers and heptamers. After cross-linking with glutaraldehyde to stabilize the oligomeric protein, hexamers and heptamers can be distinguished either by negative stain electron microscopy or electrophoretic analysis using polyacrylamide gels. We find that hexamers predominate in the presence of either dTTP or β,γ-methylene dTTP whereas the ratio between hexamers and heptamers is nearly the converse in the presence of dTDP. When formed, heptamers are unable to efficiently bind either single-stranded DNA or double-stranded DNA. We postulate that a switch between heptamer to hexamer may provide a ring-opening mechanism for the single-stranded DNA binding pathway. Accordingly, we observe that in the presence of both nucleoside di- and triphosphates the gene 4 protein exists as a hexamer when bound to single-stranded DNA and as a mixture of heptamer and hexamer when not bound to single-stranded DNA. Furthermore, altering regions of the gene 4 protein postulated to be conformational switches for dTTP-dependent helicase activity leads to modulation of the heptamer to hexamer ratio.

Keywords: helicase; DNA binding; heptamer; hexamer; phosphate sensor

Introduction

Helicases are motor proteins that translocate along single-stranded nucleic acids using energy derived from nucleotide hydrolysis.[1–3] This activity facilitates many nucleic acid metabolic processes including those that require the separation of double-stranded nucleic acids into their component single strands. The gene 4 protein of bacteriophage T7 contains within its C-terminal half the six conserved amino acid sequence motifs associated with the superfamily 4 of helicases.[4] Helicases of this family, which include the replicative *Escherichia coli* DnaB helicase, assemble as ring-shaped hexamers that encircle single-stranded DNA.[5–7] In the presence of dTTP, the gene 4 protein translocates unidirectionally 5′ to 3′ by using the energy of dTTP hydrolysis.[8,9] The movement of the protein can be inhibited by a non-hydrolyzable analog such as β,γ-methylene dTTP.

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At the replication fork, hexameric helicases translocate along the lagging strand and unwind duplex DNA to expose single-stranded DNA template for the leading strand DNA polymerase. Synthetic duplex DNA substrates used to measure the unwinding activity require a 5′-single-stranded tail onto which the hexameric helicase can bind.[10] A 3′-single-stranded tail is also required in order to assure that this strand remains outside the central channel of the helicase through which the other strand passes.[11] Kinetic data suggest that DNA binding is a multiple step process,[12,13] but the exact mechanism by which the gene 4 hexamer binds to DNA is unknown.

Unlike many other hexameric helicases, such as DnaB from *E. coli* and the gene 41 protein of bacteriophage T4, gene 4 protein loads onto single-stranded DNA without the aid of a helicase loader protein.[14] In order for the gene 4 protein to load onto single-stranded DNA it must either disassemble or the ring must open, since the protein loads onto circular DNA as efficiently as it does on linear DNA.[15] Biochemical studies argue against the disassembly and reassembly of the oligomeric complex as a mechanism for ssDNA binding.[12,13] Rather, it

has been suggested that the gene 4 protein adopts a ''lock-washer'' conformation as has been observed in the crystal structure of transcription termination factor Rho[16] and in electron micrographs of bacteriophage T4 gene 41 protein,[17] and in so doing allows ssDNA access to the central channel.[12,13,18] Another mechanism for binding to DNA is suggested from studies with the RuvB protein from *Thermus thermophilus* in which the loss of one subunit from a heptamer accompanies binding to double-stranded DNA. The loss of a subunit provides a mechanism for opening the oligomeric ring providing access to the central channel for double-stranded DNA binding to the hexameric form.[19]

In the presence of dTDP, dTTP, or β,γ-methylene dTTP, the gene 4 protein assembles into oligomeric complexes that bind to single-stranded DNA.[20] Although hexamers are the predominant form of oligomerized gene 4 protein detected by electron microscopy,[5] heptamers of gene 4 protein have also been observed.[18] Furthermore, a gene 4 protein fragment lacking the primase domain crystallized as a hexamer[21] whereas a nearly full length gene 4 protein crystallized as a heptamer[18] (Figure 1). The heptamer revealed an expanded ring with dimensions suitable for accommodating double-stranded DNA in the central channel. Thus, it was proposed that the heptameric form of the gene 4 protein could translocate along double-stranded DNA. Such a model is of biological relevance, since the gene 4 protein has been proposed to translocate along double-stranded DNA when it remodels Holiday structures.[7]

Oligomerization of the gene 4 protein is nucleotide dependent.[22] However, the residues involved in the conformational changes required for the assembly of free subunits into a ring-shaped and active gene 4 protein oligomer are largely unidentified. The crystal structure of the helicase fragment showed histidine 465 contacting the γ-phosphate of the bound nucleotide in a manner consistent with this residue acting as a conformational switch by ''sensing'' the presence or absence of the γ-phosphate. Additionally, helicase motif 4 of the hexameric helicases has also been postulated to be a region with an important role in modulating the conformational changes required for dTTPase-dependent helicase activity.[23,24]

In the present study, we have investigated the role of the heptameric form of the gene 4 protein. After cross-linking of the gene 4 protein to stabilize the oligomeric form,[25] the heptamer and hexamer forms can be observed by negative staining electron microscopy or by migration on a polyacrylamide gel. Significantly, no DNA binding by the heptamer is observed. Rather, the heptamer is disfavored under conditions that promote the binding of DNA. The ratio of heptamer to hexamer can be manipulated by the presence of nucleotide di- or triphosphates, mixing nucleotides in the presence or absence of single-stranded DNA, or altering functionally important residues thought to be switches for conformational change. Our data are compatible with a model in which the loss of a subunit from the heptamer provides a ring-opening mechanism for the hexamer to bind single-stranded DNA.

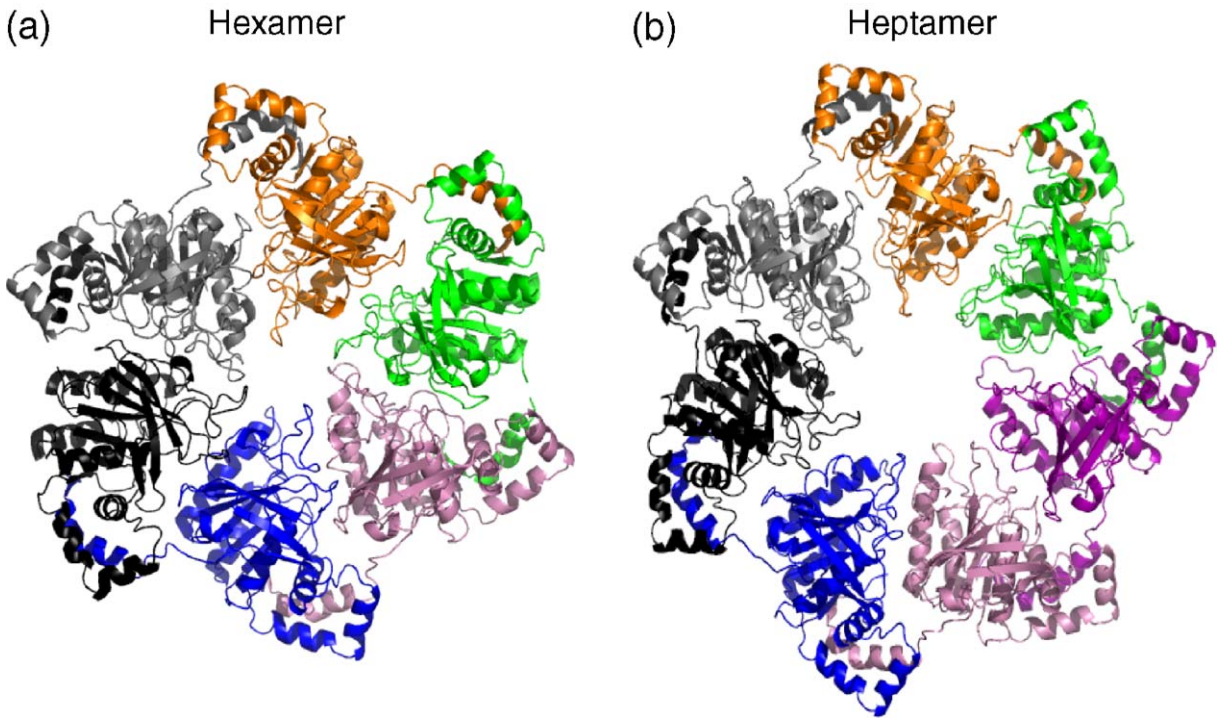

Figure 1. Crystal structures of the gene 4 protein helicase region: (a) 4D fragment (residues 241–566)[21] and (b) 56 kDa 4B helicase.[18] In each case, residues 261–549 are shown with individual subunits uniquely colored. The 4D helicase fragment crystallized as a hexamer with 2-fold symmetry while the altered 4B protein crystallized as a heptamer.

Results

Oligomeric structure of the gene 4 protein is nucleotide dependent

A representative electron microscopic image of negatively stained bacteriophage T7 gene 4 protein is shown in Figure 2(a). As previously described for the gene 4 protein,[5,18] the electron micrograph shows many ring-shaped particles. After incubation of gene 4 protein with nucleotide followed by cross-linking with glutaraldehyde to stabilize the ring structure, both heptameric and hexameric forms are observed in electron microscopic images of negatively stained samples (Figure 2(b) and (c)). Glutaraldehyde is a bi-functional compound that links covalently the amine groups of lysine in protein molecules creating a structure more stable than that attained by aggregation or oligomerization.

The ratio of heptameric to hexameric rings is dependent upon whether the protein is incubated with thymidine diphosphate (dTDP) or triphosphate (dTTP) (Table 1). In the presence of dTDP, electron microscopy shows that approximately four out of five ring structures formed by the gene 4 protein are heptameric. Conversely, the ratios of heptameric to hexameric rings are reversed when the gene 4 protein is incubated with either dTTP or β,γ-methylene dTTP. These observations are in contrast to the observations made by Toth *et al.*[18] where, in the absence of cross-linking, the type of nucleotide had no effect on the percentage of heptameric rings.

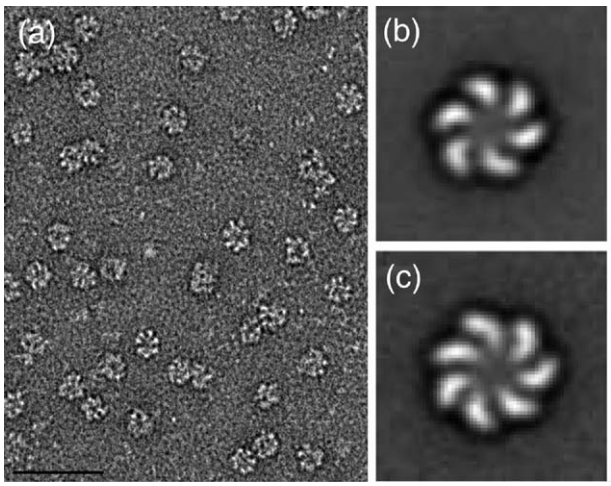

Figure 2. Electron micrograph and representative projection averages of negatively stained gene 4 protein. (a) A typical micrograph area of negatively stained gene 4 protein shows rings with six and seven subunits. The bar represents 50 nm. (b) A representative average of hexameric rings. (c) A representative average of heptameric rings. Averages were obtained by multi-reference alignment and classification. Each averaged image contains over ~500 particles. Side length of the average images is 28 nm.

Table 1. Effect of nucleotide on the ratio of heptamer to hexamer

Nucleotide	Particles	Heptamer (%)	Hexamer (%)
dTDP	3172	79	21
dTTP	3175	29	71
β,γ-Methylene dTTP	2714	17	83

The number of hexamers and heptamers was determined using the display program WEB.[41]

Oligomerization of the T7 gene 4 protein is routinely determined by polyacrylamide gel electrophoresis under non-denaturing conditions.[22,23] Frequently, multiple bands appear at sizes greater than 300 kDa (Figure 3, lane 1), but their significance has not been evaluated other than to verify oligomerization. The ability of the gene 4 protein to form both heptamers and hexamers as revealed by the crystal structure of the 56 kDa gene 4 protein 18 suggests that the two discrete but closely associated protein bands seen in the non-denaturing gel in Figure 3 represent the heptameric and hexameric forms of the enzyme.

To explore potential roles of the heptamer, polyacrylamide gel electrophoresis was used in an attempt to observe both oligomeric states under various conditions. Again, glutaraldehyde was used as a cross-linking agent so that oligomerized gene 4 protein would remain stable during electrophoresis . In Figure 3, lanes 2–5 clearly show the two protein bands or doublet resulting from oligomerized gene 4 protein in the absence of single-stranded DNA similar to those seen in the non-denaturing gel. Changes in the amount of the

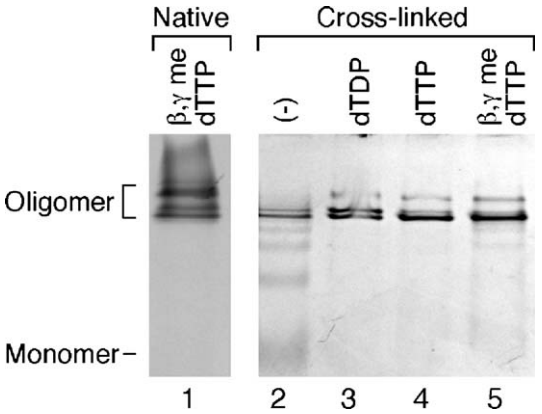

Figure 3. Gel analysis of T7 gene 4 protein oligomer. Purified gene 4 proteins were examined for their ability to oligomerize using protein electrophoresis. The non-denaturing 10% Tris-HCl/gel (native) shows 0.8 μM gene 4 protein incubated with β,γ-methylene dTTP (lane 1). In the 10% TBE/gel, cross-linked 0.8 μM gene 4 protein was incubated in the absence of nucleotide (lane 2) or in the presence of dTDP, dTTP, or β,γ-methylene dTTP without single-stranded DNA (lanes 3–5). After incubation with or without nucleotide, the gene 4 protein was cross-linked with 0.033% glutaraldehyde prior to being loaded and run on the gel. The locations of protein bands corresponding to the oligomeric and monomeric states are labeled.

slower migrating protein band of the doublet are noticeably dependent on nucleotide such that the slower migrating protein band diminishes as the nucleotide is varied from dTDP (lane 3) to dTTP (lane 4) to β,γ-methylene dTTP (lane 5). Although the protein bands are much fainter, the doublet is present in the absence of nucleotide as well (lane 2). The previous observation made by electron microscopy that heptameric ring formation is nucleotide dependent suggests that that the faster migrating band, which is the predominant species in the presence of thymidine triphosphates, represents the hexamer while the slower migrating band is heptamer. A third very large oligomeric form was observed that may represent a dodecamer. Although we cannot exclude that the higher molecular mass complex is a double heptamer, the fact that the higher molecular mass complex binds single-stranded DNA suggests that the complex is a double hexamer. Recently, it has been observed that a double hexamer of DnaB is able to simultaneously unwind two duplexes necessitating interaction between the two hexamers by essentially creating a dodecameric DNA pump.[26]

Heptamers do not bind DNA

Whether in the absence (Figure 3) or presence of single-stranded DNA (Figure 4), the doublets representing heptamers and hexamers of the gene 4 protein oligomerized in the absence of nucleotide (lanes 1) or in the presence of dTDP or dTTP (lanes 2 and 3) appear very similar. However, the protein band representing the heptamer is absent when gene 4 protein is oligomerized with single-stranded DNA and β,γ-methylene dTTP (lane 4).

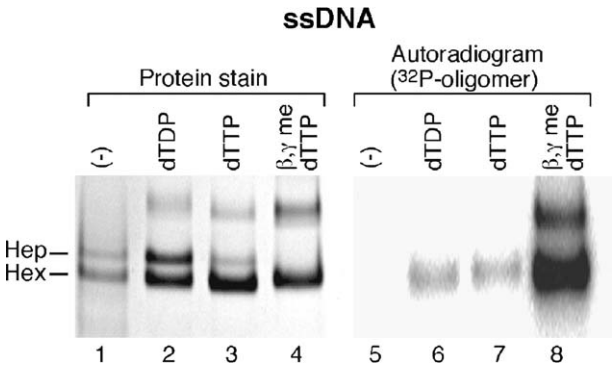

Figure 4. The oligomeric states and protein–DNA complexes formed in the presence of 50-mer oligonucleotide. The 0.8 μM gene 4 protein was incubated with no nucleotide (lanes 1 and 5), dDTP (lanes 2 and 6), dTTP (lanes 3 and 7), and β,γ-methylene dTTP (lanes 4 and 8) in the presence of 50-mer oligonucleotide prior to cross-linking. Oligomers were separated on a 10% TBE/gel after cross-linking with 0.033% glutaraldehyde. Lanes 1–4 show the location of the stained protein. Lanes 5–8 are an autoradiogram showing the position of the [^{32}P]oligonucleotide bound to gene 4 protein. Both gels have been magnified to include only the area that contained the heptamer and hexamer protein band to more clearly visualize the ratio between the two protein bands. The whole protein and single-stranded DNA binding gels can be found in Supplementary Data, Figure 1.

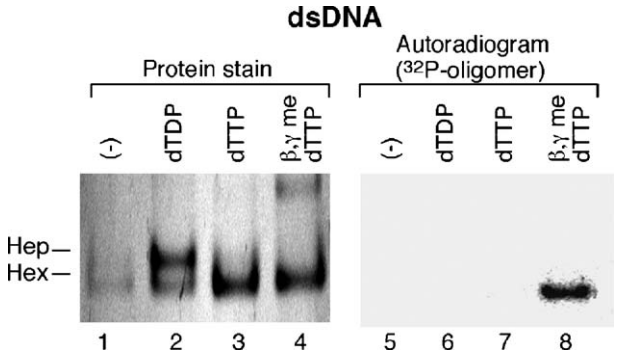

Figure 5. The oligomeric states and protein–DNA complexes formed by gene 4 protein in the presence of double-stranded DNA. The duplex DNA was the 50-mer of Figure 3 annealed to its complement as described in Materials and Methods. The 0.8 μM gene 4 protein was incubated with no nucleotide (lanes 1 and 5), dDTP (lanes 2 and 6), dTTP (lanes 3 and 7), and β,γ-methylene dTTP (lanes 4 and 8) in the presence of duplex oligonucleotide prior to cross-linking. Oligomers were sperated on a 10% TBE/gel after cross-linking with 0.033% glutaraldehyde. Lanes 1–4 show the location of the stained protein. Lanes 5–8 are an autoradiogram showing the position of the ^{32}P-labeled duplex DNA bound to gene 4 protein. Both gels have been magnified to include only the area that contained the heptamer and hexamer protein band to more clearly visualize the ratio between the two protein bands. The whole protein and double-stranded DNA binding gels are found in Supplementary Data, Figure 2.

To determine whether the heptamer is capable of binding single-stranded DNA, radiolabeled single-stranded DNA was used for electrophoretic mobility shifts. In Figure 4, lanes 5–8 are an audioradiogram of the polyacrylamide gel containing gene 4 protein oligomerized in the presence of radiolabeled single-stranded DNA with or without various nucleotides. When compared to the protein bands in lanes 1–4, it is apparent that the DNA binds to the hexamer but not the heptamer. As observed previously,[15,20] gene 4 protein does not bind single-stranded DNA in the absence of nucleotide (lanes 1 and 5) and also the protein binds single-stranded DNA weakly when dTDP is present (lanes 2 and 6). The lower affinity of gene 4 protein for single-stranded DNA in the presence of dTDP may be partially due to the abundance of the heptameric form of the enzyme under such conditions. The weak binding of gene 4 protein to single-stranded DNA in the presence of dTTP (lanes 3 and 7) is most likely due to the protein translocating off the 3′ end of the short oligonucleotide used in the reaction. In the presence of β,γ-methylene dTTP (lanes 4 and 8), nearly all of the single-stranded DNA is bound to oligomerized gene 4 protein.

In order to examine the ability of the oligomeric form of the gene 4 protein to bind duplex DNA we repeated the experiment with a 50-mer duplex DNA

replacing the single-stranded oligonucleotide (Figure 5). The oligomerization of gene 4 protein in the presence of double-stranded DNA with or without various nucleotides (lanes 1–4), is nearly identical to that previously observed for single-stranded-DNA in Figure 4. Gene 4 protein binds poorly to double-stranded DNA.[20] However, due to the reported DNA remodeling ability of the gene 4 protein[7] it has been postulated that the heptameric form of the protein may possess the ability to translocate along double-stranded DNA.[18] However, in the presence of radiolabeled double-stranded DNA with or without various nucleotides, the heptameric form of the protein does not bind double-stranded DNA (lanes 5–8). A small amount of duplex DNA is observed to bind gene 4 protein in the presence of β,γ-methylene dTTP but compared to that seen with single-stranded DNA (Figure 4) it is relatively insignificant.

Single-stranded DNA dependent hexamer formation

The ratio of heptamers to hexamers formed in the presence of nucleotide is not significantly changed in the absence or presence of single-stranded DNA (Figures 4 and 5). Since the gene 4 protein is active in the presence of dTTP, it is difficult to detect any switching between the heptameric and hexameric states. Therefore, in order to obtain a snapshot picture of the oligomeric states of the protein during its hydrolysis cycle, dTDP and β,γ-methylene dTTP were added at different molar ratios such that

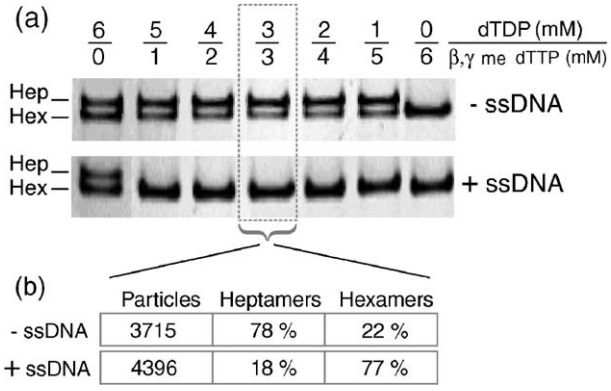

Figure 6. Effect of single-stranded DNA on oligomeric conformations induced by mixed nucleotides. (a) Absence of 50-mer oligonucleotide and 150 nM 50-mer oligonucleotide. The lanes show gene 4 protein incubated with the indicated ratios of β,γ-methylene dTTP to dTDP. After incubation with the indicated ratio of β,γ-methylene dTTP to dTDP, the samples were cross-linked with 0.033% glutaraldehyde and run on a 10% TBE /gel to separate the oligomers. Both gels have been magnified to include only the area that contained the heptamer and hexamer protein band to more clearly visualize the ratio between the two protein bands. (b) The observed dependence of heptamer formation on the absence of single-stranded DNA when gene 4 protein is incubated with mixed nucleotides is confirmed by electron microscopy. Samples of gene 4 protein were negatively stained as for Figure 2.

oligomers would contain diverse arrangements of nucleotides (Figure 6(a)). In the absence of single-stranded DNA, increasing the amount of β,γ-methylene dTTP in comparison to dTDP has little effect on the ratio of heptamer to hexamer until there is no dTDP present. However, a switch from heptamer to hexamer occurs which is dependent on the presence of single-stranded DNA under the same concentrations of nucleotide. When approximately one subunit per oligomer contains β,γ-methylene dTTP *versus* dTDP the gene 4 protein oligomerizes as mostly hexamer. Conversely, approximately one subunit per oligomer containing dTDP *versus* β,γ-methylene dTTP allows for the formation of the heptamer when single-stranded DNA is absent. Sorting of electron microscopic images of gene 4 protein incubated with equal amounts of dTDP and β,γ-methylene dTTP illustrates that heptamers are disfavored in the presence of DNA under this condition (Figure 6(b)).

Alterations in the gene 4 protein influence the ratio of heptamer and hexamer

Histidine 465 contacts the γ-phosphate of the bound nucleotide triphosphates in a manner consistent with this residue acting as a conformational switch or a sensor for the γ-phosphate of the bound nucleotide.[21] The positionally conserved residues in *Bacillus subtilis* DnaB and the replicative helicase RepA encoded by plasmid RSF1010 are a glutamine and histidine, respectively. In these enzymes, site-directed mutagenesis studies have shown that altering this residue decreases helicase activity while nucleotide hydrolysis activity and affinity for single-stranded DNA are only slightly affected.[27,28]

Since the ratio of heptameric to hexameric rings is dependent on the presence or absence of the γ-phosphate, altering the residue at position 465 should have an effect on this ratio if histidine 465 is a sensor capable of distinguishing dTTP from dTDP. Consequently, histidine 465 was changed to leucine and tyrosine so that position 465 is occupied by a residue either unable to detect the γ-phosphate of bound dTTP due to the lack of a functional group (gp4-H465L) or inaccurately detect a γ-phosphate for bound dTDP because of a larger functional group (gp4-H465Y). As shown in Figure 7(a), these altered gene 4 proteins retain their ability to bind single-stranded DNA. However, gp4-H465Y is devoid of single-stranded DNA dependent dTTPase activity and gp4-H465L has significantly decreased activity as compared to wild-type gene 4 protein (Figure 7(b)). The oligomerization of gp4-H465L and gp4-H465Y was examined as described previously for wild-type gene 4 protein. In the absence of single-stranded DNA, both of the altered gene 4 proteins oligomerized as heptamers and hexamers independent of the type of nucleotide present unlike the wild-type protein (Figure 7(c)). Thus, gp4-H465L (lanes 5–8) and gp4-H465Y (lanes 9–12) have lost the ability to distinguish nucleoside triphosphates from nucleoside diphosphates in the absence of single-

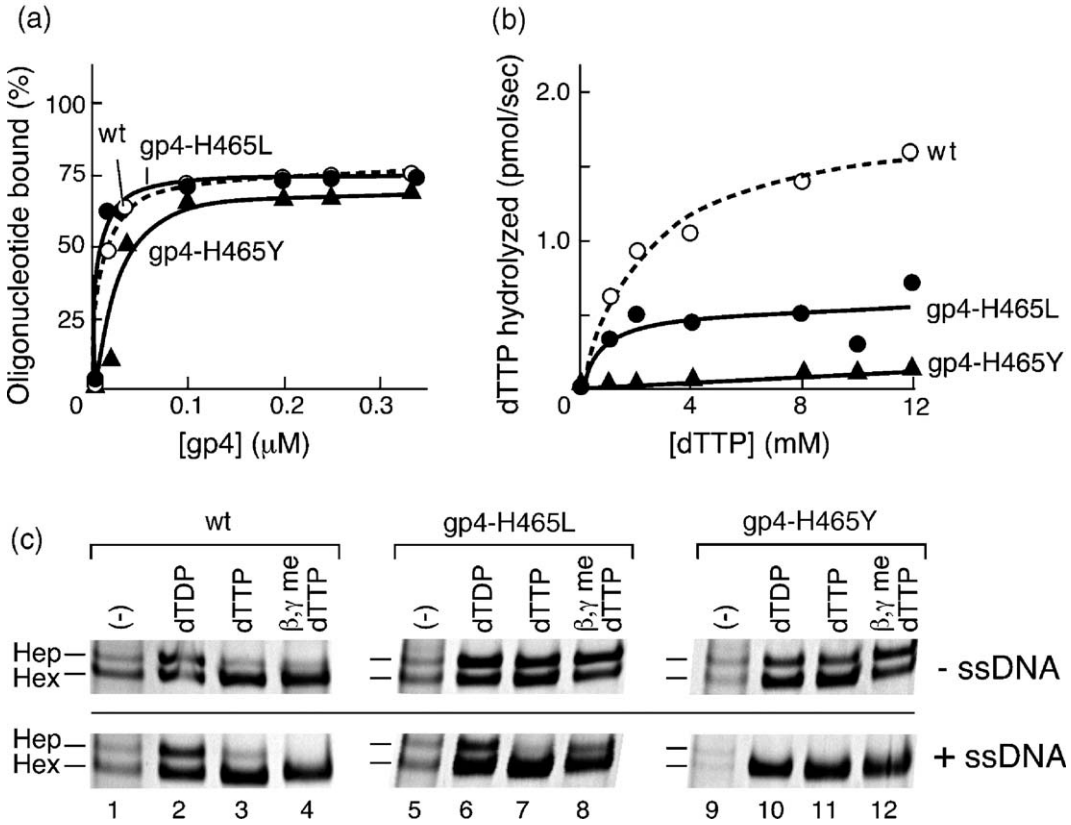

Figure 7. Histidine 465 is essential for dTTP hydrolysis and acts as a phosphate sensor. (a) Single-stranded DNA-binding affinity of gene 4 protein measured by nitrocellulose filter binding. Each reaction contained 1 nM of (5′-^{32}P)-labeled 50-mer oligonucleotide, the indicated amount of either wild-type gene 4 protein (○), gp4-H465L(■), or gp4-H465Y (▼) (0–0.3 μM), and 5 mM β,γ-methylene 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. Each point represents the mean of three or more independent experiments. (b) Single-stranded DNA-dependent dTTP hydrolysis catalyzed by gene 4 proteins. Gene 4 protein catalyzes the single-stranded DNA-dependent hydrolysis of dTTP to dTDP and Pi. Each 10 μl reaction contained 1.1 nM M13 single-stranded DNA, the indicated concentration of dTTP (0.5, 1, 2, 4, 6, 8 mM), and 0.1 μCi of [α-^{32}P]dTTP, and 120 nM of wild-type gene 4 protein (○), gp4-H465L (■), or gp4-H465Y (▼). 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 Materials and Methods. The rate of dTTP hydrolysis is plotted against the initial concentration of dTTP. Each point represents the mean of three or more independent experiments. (c) Oligomerization of T7 gene 4 proteins after cross-linking. The 0.8 μM of the altered gene 4 proteins was incubated in the absence of nucleotide (lanes 5 and 9) or in the presence of dTDP (lanes 6 and 10), dTTP (lanes 7 and 11), or β,γ-methylene dTTP (lanes 8 and 12) in the absence or presence of single-stranded DNA. After incubation, the gene 4 protein was cross-linked with 0.033% glutaraldehyde and run on a 10% TBE/gel. The protein gels of wild-type gene 4 protein from Figures 3 and 4 are shown for comparison (lanes 1–4).

stranded DNA. When gp4-H465L binds single-stranded DNA in the presence of β,γ-methylene dTTP, the polyacrylamide gel reveals the presence of some heptamer (lane 8). In addition, gp-H465Y does not form heptamers when both dTDP and single-stranded DNA are present (lane 10). These results suggest that when single-stranded DNA is bound to the altered proteins that gp4-H465L has difficulty ''sensing'' the γ-phosphate while gp4-H465Y detects a phosphate even when the γ-phosphate is absent.

Helicase motif 4 (residues 480–500)[29] has also been implicated as a conformational switch region of the gene 4 protein.[23,24] T7 bacteriophage submitted to random mutagenesis identified arginine 493 as an important residue. Mutant phage with arginine 493 changed to a glutamine can not complement for the growth of T7Δ4-1 (data not shown). As shown in Figure 8(a), gp4-R493Q has lost its ability to bind single-stranded DNA. In a manner consistent with arginine 493 being located within a conformational switch region, gp4-R493Q forms both heptamers and hexamers under all conditions (Figure 8(b)).

Discussion

The T7 gene 4 protein has been observed previously to form hexamers and heptamers as well as larger oligomers.[5,18,30,31] However, identity of oligomers of six or seven subunits is beyond the resolution of high pressure gel-filtration[22] and native polyacrylamide gels have been inconclusive.[20,23] Here we show that the oligomeric states of the gene 4 protein can be identified using polyacrlyamide gel electrophoresis

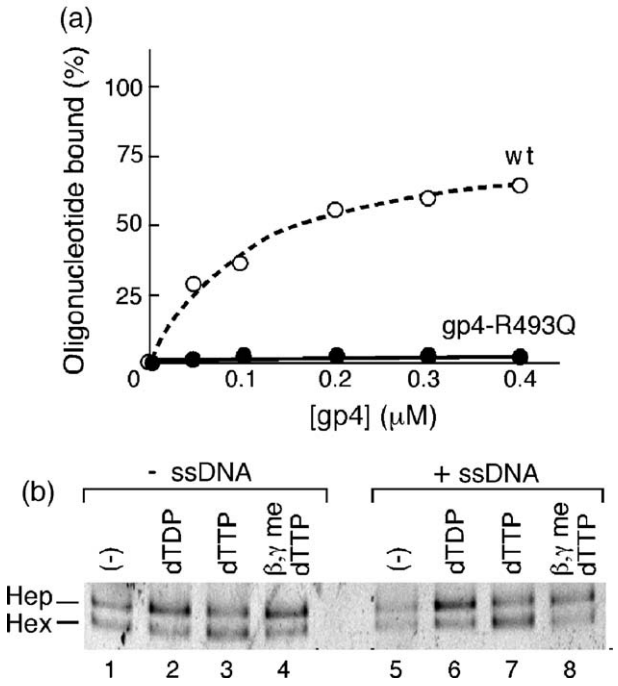

Figure 8. Arginine 493 modulates the oligomeric state of gene 4 protein. (a) Single-stranded DNA-binding affinity of gene 4 protein measured by nitrocellulose filter binding. Each reaction contained 1 nM of (5′-^{32}P)-labeled 50-mer oligonucleotide, the indicated amount of either wild-type gene 4 protein (○) or gp4-R493Q (♦) (0–0.4 μM), and 5 mM β,γ-methylene 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. Each point represents the mean of three or more independent experiments. (b) Oligomerization of T7 gene 4 proteins after cross-linking. The 0.8 μM of the altered gene 4 proteins was incubated in the absence of nucleotide (lanes 1 and 5) or in the presence of dTDP (lanes 2 and 6), dTTP (lanes 3 and 7), or β,γ-methylene dTTP (lanes 4 and 8) with or without single-stranded DNA. After incubation with the indicated nucleotide, the gene 4 protein was cross-linked with 0.033% glutaraldehyde and run on a 10% TBE/gel.

following cross-linking of the subunits with glutaraldehyde (Figures 3 and 4). Here, we have used electron microscopy to assign the correct number of subunits to the protein species identified by gel electrophoresis (Figure 2).

The assignment of function to the hexamer and heptamer has been difficult. The identification of hexamer bound to single-stranded DNA suggested that the hexamer was the functional oligomeric state for translocation and unwinding of DNA.[5] The larger central cavity of the heptamer[18] raised the possibility that this species could bind to duplex DNA and function as the active species in the remodeling of Holiday junctions.[7] Here, we show that formation heptamers and hexamers by the T7 gene 4 protein is dependent upon whether nucleoside di- or triphosphates are bound to the enzyme. Under conditions favorable for the formation of heptamers, the gene 4 protein does not bind single-stranded or double-stranded DNA (Figures 4 and 5). Rather, the heptameric is absent when the gene 4 protein is in the presence of either dTTP or β,γ-methylene dTTP. In the presence of both nucleoside di- and triphosphates, conversion between the two oligomeric forms is single-stranded DNA dependent (Figure 6).

Polyacrylamide gels of gene 4 protein cross-linked in the absence of nucleotide show only modest oligomerization (Figures 3 and 4). This result corroborates previous observations that oligomerization is nucleotide dependent.[22,30,31] The catalytically competent state is thought to be represented by gene 4 protein in the presence of the non-hydrolyzable analog β,γ-methylene dTTP. This complex has been shown to be a hexamer that is stably bound to single-stranded DNA located within the central channel.[5,32] We show that gene 4 protein incubated with dTTP and β,γ-methylene dTTP forms heptamers poorly and hexamers predominate even in the absence of single-stranded DNA (Table 1; Figures 3 and 4). In the presence of dTDP, heptamers and hexamers formed with perhaps a slight preference for heptamer formation. The low affinity of the gene 4 protein for single-stranded DNA in the presence of dTDP has been incorporated into the mechanisms of almost all models of how dTTP hydrolysis is coupled to DNA unwinding.[3] Our observation that much of the gene 4 protein exists as a heptamer in the presence of dTDP necessitates that these models be revisited.

We show that the oligomerization state of the gene 4 protein is dependent upon the presence of a γ-phosphate on the bound nucleotide. In the crystal structure of the 4D helicase fragment, histidine 465 contacts the γ-phosphate of the bound nucleotide.[29] When histidine 465 is changed to either a leucine or a tyrosine the ability of the enzyme to distinguish dTTP from dTDP is altered as determined by the ratio of hexamers to heptamers formed by gp-H465L and gp4-H465Y. We conclude that histidine 465 does function as a ''phosphate sensor''. Furthermore, gp-H465L and gp4-H465Y show decreased dTTPase activity suggesting that this residue is also important for the catalytic mechanism of the gene 4 protein.

Another region suggested to be a conformational switch in the gene 4 protein is helicase motif 4 (residues 480–500).[29] A gene 4 protein altered in this region, gp4-R487A, was found to have a single-stranded DNA-dependent dTTPase activity lower than the dTTPase activity observed in the absence of single-stranded DNA.[23] Arginine 493 is also located within the helicase motif 4. The altered gene 4 protein gp4-R493Q is unable to form exclusively hexamers in the presence of nucleoside triphosphates (Figure 8). The inability of gp4-R493Q to bind single-stranded DNA may possibly be the result of a deficiency in the switching between oligomeric states. Like gp4-H465L and gp4-H465Y, gp4-R493Q is indifferent to nucleotides in the absence of single-stranded DNA. However, gp4-R493Q is unable to bind to single-stranded DNA

and, thus, also exhibits no nucleotide dependence of heptamer and hexamer ratios in the presence of single-stranded DNA. This result further shows binding single-stranded DNA plays a role in hexamer formation albeit secondary to nucleotide binding. The modification of oligomer formation observed when conformation switch regions of the gene 4 protein are altered suggests that heptamer formation may be an important step in the formation of hexamers bound to single-stranded DNA.

Our finding that heptamers cannot bind either single or double-stranded DNA could be used as an argument that heptamers arise as a result of a lax assembly of identical subunits. Consequently, heptamers would have no biological significance and their fate would be dictated by their equilibrium with the functional hexamers to which they can clearly be converted. On the other hand, it is also plausible that the heptamer of gene 4 protein is an essential intermediate in the pathway for loading of gene 4 protein onto single-stranded DNA. A topological problem exists for binding any preformed ring to DNA other than threading the ends of the DNA through the ring. The later mode of loading is clearly not essential for the gene 4 protein, since it loads onto circular M13 DNA as well as onto linear DNA.[15] Alternatively, the oligomeric protein must disassemble and then reassemble onto the DNA or as previously proposed for the gene 4 protein based on kinetic data[12,13] the ring would transiently open to allow for loading onto DNA. It is worth noting that the structurally related hexameric helicases DnaB of *E. coli* and gene 41 protein of T4 bacteriophage make use of helicase loader proteins to efficiently assemble on single-stranded DNA.[14] Although the T7 gene 4 protein loads onto DNA without the aid of a helicase loader, it has been suggested that the primase region of the bi-functional gene 4 protein provides the loading function.[13,33]

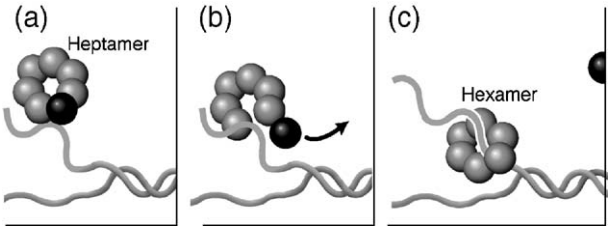

Figure 9. A ring-opening model for the loading of the T7 DNA helicase onto single-stranded DNA. The heptameric form of the gene 4 protein may be involved in the ''ring-opening mechanism'' of DNA binding suggested by biochemical studies.[12,13] In this model, the T7 gene 4 primase/helicase consists of an equilibrium mixture of hexamers and heptamers. Upon encountering single-stranded DNA (a) one of the subunits of the heptamer, most likely the one contacting the DNA, undergoes a conformational change leading to the release (b) of that subunit. The loss of the subunit transiently opens the ring allowing the simultaneous entry of the single-stranded DNA into the central binding cavity (c) where it is then bound tightly by the DNA binding loops that protrude into the central core.[21,34]

The hydrolysis of dTTP in the presence of single-stranded DNA occurs sequentially around the ring.[34,35] It is likely that the catalytically competent state of the enzyme consists of hexamers with both dTTP and dTDP bound to various subunits simultaneously. The gene 4 protein in this state would exist as a hexamer when bound to single-stranded DNA and as a mixture of heptamer and hexamer when not bound to single-stranded DNA (Figure 6). Thus, one mechanism for loading the gene 4 protein onto DNA envisions the loss of a subunit from the heptamer upon contact with single-stranded DNA with a concomitant opening of the ring to allow passage of the DNA into the central cavity (Figure 9). Closure of the hexamer around the DNA then completes the loading process. Such a ring-opening *via* loss of a subunit has been proposed for the MCM protein from *Mehanobacterium thermoautotrophicum*[36] based on its existence as a heptamer in the absence of DNA. Yet another helicase, the RuvB protein from *E. coli* , also exists as a mixture of hexamers and heptamers in the absence of DNA but only as hexamers in the presence of DNA.[19]

On the assumption that subunit loss from the heptamer underlies the ring-opening mechanism the question then arises as to the identity of the trigger that leads to the release of a subunit. It has been postulated that the initial binding of the gene 4 protein occurs *via* the primase domain of the protein,[13,33] since the DNA binding and catalytic sites for primase activity resides on the outside of the oligomeric ring.[37] How this initial contact of the gene 4 protein *via* the primase domain would trigger subunit release is speculative. No structure of the gene 4 protein bound to DNA is available. The crystal structure of the hexamer consists only of the helicase domain and the hexameric structure lacks the Cys4 zinc motif of the primase domain. Perhaps the presence of the primase domain in the heptameric structure is precisely the reason this form of the protein crystallized as a heptamer in the absence of DNA. Interestingly, the heptameric structure shows the primase domains of the subunits splayed out from the tightly packed heptamer like the fingers of an upraised hand making them available for DNA binding. Toth *et al.*[18] suggested that the binding of single-stranded DNA to the primase region leads to a conformational change that allows the heptameric ring to open through the loss of contacts at the subunit-to-subunit interface. One can envision that the conformational change that arises upon DNA binding places constraints on the helicase domain and shifts the equilibrium from a seven to a six-membered ring.

Materials and Methods

Materials

Radiolabeled nucleotides were purchased from GE Healthcare. Polynuclucleotide kinase was purchased from New England Biolabs. Nucleotides (dTTP, dTDP,

and β,γ-methylene dTTP) were purchased from USB Corp. Polyacrylamide gels were from Bio-Rad. E. coli strain HMS174(DE3) was from Novagen. Plasmid 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 co-production of the 56 kDa protein.[38]

Construction of plasmids

Mutations of histidine 465 were introduced into pET11gp4-63 using the Quickchange II Mutagenesis kit (Stratagene) and the following primers: H465L(+) 5′-CTG-GTCGTAATTTGT*CT*CCTTAAGAACCCAG AC-3′, H465L (−) 5′-GTCTGGGTTCTTAAG*GAG*ACCAAATTACGAC-CAG-3′, H465Y(+) 5′-CTGGTCGTAATTTGT*TAC*CTTAA-GAACCCAGAC-3′, H465L(−) 5′-GTCTGGGTTCTTAAG-*GTA*ACCAAATTACGACCAG-3′. The mutated gene 4-coding regions (underlined sequence in the oligonucleotide) were confirmed by DNA sequence analysis. The mutation of arginine 493 changed to a glutamine was introduced into pET24gp4-63 using the mega primer method. A PCR product was prepared with a mutagenic primer (R493Q 5′-GCA CTA CAG CAA CTA TCT GAT AC-3′) and a primer outside the downstream coding region (Hin 5′-TTAGCAG-CCGGATCTCAGTGG-3′) using pET24-gp4 as a template. The isolated PCR product(1.2 kb) was used as ''mega'' primer together with an outside primer upstream of the coding region (Nde 5′-TACTGTTGATGGGTGTCT GGTC-AG-3′) to generate PCR product containing the mutated gene 4. The second PCR product was isolated and cut with HindIII and NdeI. The resulting 2.7 kb DNA was ligated with pET24-gp4 previously cut with the same restriction enzymes. T7Δ4-1, a phage in which the gene 4 coding region has been deleted, can grow in *E. coli* cells harboring a plasmid that expresses a functional gene 4.[38] A plasmid expressing the gp4-R493Q protein was transformed into *E. coli* C600. The host cells were then infected with T7Δ4-1. The plasmid containing the glutamine substitution of arginine 493 did not support the growth of T7Δ4-1.

Protein overproduction and purification

Wild-type and recombinant gene 4 proteins used for polyacrylamide gel electrophoresis and biochemical assays were purified following procedures described.[39] *E. coli* strain HMS 174(DE3), transformed with the plasmid expressing gene 4 protein, was grown to an A_{600} of 1 in LB medium. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were cultured for three additional hours and then harvested by centrifugation. Harvested cells were washed in STE buffer (10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA), collected by centrifugation, and then resuspended in buffer L (20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride) and subjected to three cycles of freezing and thawing in the presence of 0.2 mg/ml of lysozyme. The lysed cells were centrifuged at 15,000***g*** for 30 min and polyethylene glycol (Fluka; PEG4000) was added to a final concentration of 10%. The PEG pellet was collected by centrifugation at 5000***g*** for 20 min, resuspended in buffer P (20 mM potassium phosphate (pH 6.8), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol), and loaded onto a phosphocellulose column (Whatman). Protein was eluted with a KCl gradient from 0.02 M to 1 M, and fractions containing gene 4 protein were combined. Fractions containing gene 4 protein were identified by gel analysis of an aliquot of each fraction. The pooled fractions were diluted twofold with buffer AB (20 mM potassium phosphate(pH 6.8), 20 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 0.5 M KCl) and then loaded onto an ATP-agarose affinity column (Sigma Chemical Co.) and eluted with buffer AE (20 mM potassium phosphate (pH 6.8), 20 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.5 M KCl). Finally, the purified protein was dialyzed against storage buffer (20 mM potassium phosphate (pH 7.5), 0.1 mM DTT, 0.1 mM EDTA, 50% glycerol) and stored at −20 °C. Wild-type gene 4 protein used in electron microscopy experiments was submitted to the identical purification procedure except that glycerol was absent from all steps.

Native gel electrophoresis of gene 4 protein

The ability of gene 4 protein to form oligomers under native conditions was determined by analyzing the protein on a pre-cast, non-denaturing 10% Tris–HCl/polyacrylamide gel (Bio-Rad). Each reaction contained 0.8 μM protein, 1 mM β,γ-methylene dTTP, 2.5 μM 50-mer oligonucleotide, 40 mM Tris-HCl (pH 7.5), 10 mM MgCl, 10 mM DTT, and 50 mM potassium glutamate. After incubation at 37 °C for 30 min, the protein was loaded on to gel and electrophoresis was carried out at 8 V/cm and 4 °C with 0.25×TBE as the running buffer. After staining with Bio-safe-Coomassie (Bio-Rad), oligomerization of gene 4 proteins was verified by protein bands of greater than 63 kDa.[23]

Cross-linking of gene 4 protein

The cross-linking of gene 4 protein was as performed by the method of Yong and Romano[25] with minor changes. The reactions (10 μl) were carried out in 40 mM Tris–HCl (pH 7.5), 10 mM MgCl, 10 mM DTT, and 50 mM potassium glutamate. Each reaction contained 0.8 μM gene 4 protein with or without 150 nM oligonucleotide (5′-ATGACCATGATTTCGACGTTTTTTTTTT-TGGGGATCCTC TAACCTGCGCA-3′) or duplex DNA and either no nucleotide, dTDP, dTTP, or β,γ-methylene dTTP as indicated. Duplex DNA was prepared by annealing the 50-mer (5′-ATGACCATGATTTCGACG-TTTTTTTTTTTGGGGATCCTCTAACC TGCGCA-3′) with its complement (5′-TGCGCAGGTTAGAGGATC CC-CAAAAA AAAAAACGTCGAAATCATGGTCAT-3′) in 0.1 M NaCl. The reaction mixtures were incubated at 37 °C for 30 min, after which, 2 μl of 0.2%(v/v) glutaraldehyde solution was added to each reaction. The reactions were incubated for a further 30 s and then stopped by the addition of 5 μl of 100 mM EDTA for electron microscopy or 5 μl of 100 mM EDTA, 40% glycerol, 0.1% (w/v) bromophenol blue, and 0.1%(w/v) xylene cyanol for electrophoresis.

Electrophoresis of cross-linked gene 4 protein

The cross-linked proteins (16 μl) were loaded on to a pre-cast 10% non-denaturing polyacryalmide/TBE gel (Bio-Rad). Electrophoresis was carried out at 8 V/cm and 4 °C with 0.25×TBE as the running buffer. After electrophoresis, the polyacrylamide gel was stained with Bio-Safe Coomassie (Bio-Rad) to visualize the protein bands.

Gel retardation of bound DNA

The DNA-binding reactions were preformed as described above with the only difference being that 150 nM of ^{32}P-labeled oligonucleoitde or ^{32}P-labeled duplex DNA replaced unlabeled oligonulceotide. After cross-linking, the stopped reactions (16 μl) were loaded on to a pre-cast 10% non-denaturing polyacryalmide/TBE gel (Bio-Rad). Electrophoresis was carried out at 8 V/cm and 4 °C with 0.25×TBE as the running buffer. After electrophoresis, the polyacrylamide gel was subjected to audioradiography, and the radioactivity of the individual bands was quantified by a phosphoimaging scanner (Fuji BAS 1000).

Electron microscopy and image processing

Uranyl formate stained samples were prepared for electron microscopy as described.[40] Images were taken with a Philips Tecnai T12 electron microscope at an acceleration voltage of 120 kV with a magnification of 52,000× and a defocus of 1.5 μm using low-dose procedures. After inspection with JEOL JFO-3000 laser diffractometer, drift-free images were digitized with a Zeiss SCAI scanner using a step size of 7 μm. The 3×3 pixels were averaged to yield a pixel size of 0.4 nm at the specimen level. Approximately 3000 particles were selected for each nucleotide condition using WEB, the display program associated with SPIDER program suite.[41] The helicase particles were windowed into 70×70 pixel images and subjected to ten rounds of multi-reference alignment and K-means classification specifying 15 output classes.

DNA binding assay

DNA binding affinity of gene 4 protein was measured by nitrocellulose filter binding. The reaction (10 μl) containing 1 nM of (5′-^{32}P)-labeled 50-mer oligonucleotide (5′-ATGACCATGATTTCGACGTTTTTTTTTTGGGGATCCTCTAACCTGCGCA-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 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.

dTTPase assay for gene 4 protein

Gene 4 protein catalyzes the single-stranded DNA-dependent hydrolysis of dTTP, a reaction coupled to its translocation on ssDNA.[31] The reaction (10 μl) contained 1.1 nM M13 single-stranded DNA, dTTP at the concentration indicated in the text (0.1 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 80 nM gene 4 protein. After incubation at 37 °C for 30 min 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 nucleotide triphosphates from diphosphates. The amount of [α-^{32}P]dTDP formed was analyzed using a Fuji BAS 1000 Bioimaging analyze.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.05.037

References

1. Lohman, T. M. & Bjornson, K. P. (1996). Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214.
2. Matson, S. W. & Kaiser-Rogers, K. A. (1990). DNA helicases. *Annu. Rev. Biochem.* **59**, 289–329.
3. Patel, S. S. & Picha, K. M. (2000). Structure and function of hexameric helicases. *Annu. Rev. Biochem.* **69**, 651–697.
4. Hall, M. C. & Matson, S. W. (1999). Helicase motifs: the engine that powers DNA unwinding. *Mol. Microbiol.* **34**, 867–877.
5. Egelman, E. H., Yu, X., Wild, R., Hingorani, M. M. & Patel, S. S. (1995). Bacteriophage T7 helicase/primase proteins form rings around single-stranded DNA that suggest a general structure for hexameric helicases. *Proc. Natl Acad. Sci. USA*, **92**, 3869–3873.
6. Hacker, K. J. & Johnson, K. A. (1997). A hexameric helicase encircles one DNA strand and excludes the other during DNA unwinding. *Biochemistry*, **36**, 14080–14087.
7. Kaplan, D. L. & O'Donnell, M. (2002). DnaB drives DNA branch migration and dislodges proteins while encircling two DNA strands. *Mol. Cell*, **10**, 647–657.
8. Tabor, S. & Richardson, C. C. (1981). Template recognition sequence for RNA primer synthesis by gene 4 protein of bacteriophage T7. *Proc. Natl Acad. Sci. USA*, **78**, 205–209.
9. Kim, D. E., Narayan, M. & Patel, S. S. (2002). T7 DNA helicase: a molecular motor that processively and unidirectionally translocates along single-stranded DNA. *J. Mol. Biol.* **321**, 807–819.
10. Matson, S. W., Tabor, S. & Richardson, C. C. (1983). The gene 4 protein of bacteriophage T7. Characterization of helicase activity. *J. Biol. Chem.* **258**, 14017–14024.

11. Kaplan, D. L. (2000). The 3′-tail of a forked-duplex sterically determines whether one or two DNA strands pass through the central channel of a replication-fork helicase. *J. Mol. Biol.* **301**, 285–299.
12. Picha, K. M., Ahnert, P. & Patel, S. S. (2000). DNA binding in the central channel of bacteriophage T7 helicase-primase is a multistep process. Nucleotide hydrolysis is not required. *Biochemistry*, **39**, 6401–6409.
13. Ahnert, P., Picha, K. M. & Patel, S. S. (2000). A ring-opening mechanism for DNA binding in the central channel of the T7 helicase-primase protein. *EMBO J.* **19**, 3418–3427.
14. Davey, M. J. & O'Donnell, M. (2003). Replicative helicase loaders: ring breakers and ring makers. *Curr. Biol.* **13**, R594–R596.
15. Matson, S. W. & Richardson, C. C. (1985). Nucleotide-dependent binding of the gene 4 protein of bacteriophage T7 to single-stranded DNA. *J. Biol. Chem.* **260**, 2281–2287.
16. Skordalakes, E. & Berger, J. M. (2003). Structure of the Rho transcription terminator: mechanism of mRNA recognition and helicase loading. *Cell*, **114**, 135–146.
17. Norcum, M. T., Warrington, J. A., Spiering, M. M., Ishmael, F. T., Trakselis, M. A. & Benkovic, S. J. (2005). Architecture of the bacteriophage T4 primosome: electron microscopy studies of helicase (gp41) and primase (gp61). *Proc. Natl Acad. Sci. USA*, **102**, 3623–3626.
18. Toth, E. A., Li, Y., Sawaya, M. R., Cheng, Y. & Ellenberger, T. (2003). The crystal structure of the bifunctional primase-helicase of bacteriophage T7. *Mol. Cell,* **12**, 1113–1123.
19. Miyata, T., Yamada, K., Iwasaki, H., Shinagawa, H., Morikawa, K. & Mayanagi, K. (2000). Two different oligomeric states of the RuvB branch migration motor protein as revealed by electron microscopy. *J. Struct. Biol.* **131**, 83–89.
20. Hingorani, M. M. & Patel, S. S. (1993). Interactions of bacteriophage T7 DNA primase/helicase protein with single-stranded and double-stranded DNAs. *Biochemistry*, **32**, 12478–12487.
21. Singleton, M. R., Sawaya, M. R., Ellenberger, T. & Wigley, D. B. (2000). Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell*, **101**, 589–600.
22. Patel, S. S. & Hingorani, M. M. (1993). Oligomeric structure of bacteriophage T7 DNA primase/helicase proteins. *J. Biol. Chem.* **268**, 10668–10675.
23. Notarnicola, S. M., Park, K., Griffith, J. D. & Richardson, C. C. (1995). A domain of the gene 4 helicase/primase of bacteriophage T7 required for the formation of an active hexamer. *J. Biol. Chem.* **270**, 20215–20224.
24. Washington, M. T., Rosenberg, A. H., Griffin, K., Studier, F. W. & Patel, S. S. (1996). Biochemical analysis of mutant T7 primase/helicase proteins defective in DNA binding, nucleotide hydrolysis, and the coupling of hydrolysis with DNA unwinding. *J. Biol. Chem.* **271**, 26825–26834.
25. Yong, Y. & Romano, L. J. (1995). Nucleotide and DNA-induced conformational changes in the bacteriophage T7 gene 4 protein. *J. Biol. Chem.* **270**, 24509–24517.
26. Kaplan, D. L. & O'Donnell, M. (2004). Twin DNA pumps of a hexameric helicase provide power to simultaneously melt two duplexes. *Mol. Cell,* **15**, 453–465.
27. Ziegelin, G., Niedenzu, T., Lurz, R., Saenger, W. & Lanka, E. (2003). Hexameric RSF1010 helicase RepA: the structural and functional importance of single amino acid residues. *Nucl. Acids Res.* **31**, 5917–5929.
28. Soultanas, P. & Wigley, D. B. (2002). Site-directed mutagenesis reveals roles for conserved amino acid residues in the hexameric DNA helicase DnaB from *Bacillus stearothermophilus*. *Nucl. Acids Res.* **30**, 4051–4060.
29. Sawaya, M. R., Guo, S., Tabor, S., Richardson, C. C. & Ellenberger, T. (1999). Crystal structure of the helicase domain from the replicative helicase-primase of bacteriophage T7. *Cell*, **99**, 167–177.
30. Hingorani, M. M. & Patel, S. S. (1996). Cooperative interactions of nucleotide ligands are linked to oligomerization and DNA binding in bacteriophage T7 gene 4 helicases. *Biochemistry*, **35**, 2218–2228.
31. Matson, S. W. & Richardson, C. C. (1983). DNA-dependent nucleoside 5′-triphosphatase activity of the gene 4 protein of bacteriophage T7. *J. Biol. Chem.* **258**, 14009–14016.
32. Yu, X., Hingorani, M. M., Patel, S. S. & Egelman, E. H. (1996). DNA is bound within the central hole to one or two of the six subunits of the T7 DNA helicase. *Nature Struct. Biol.* **3**, 740–743.
33. VanLoock, M. S., Chen, Y. J., Yu, X., Patel, S. S. & Egelman, E. H. (2001). The primase active site is on the outside of the hexameric bacteriophage T7 gene 4 helicase-primase ring. *J. Mol. Biol.* **311**, 951–956.
34. Crampton, D. J., Mukherjee, S. & Richardson, C. C. (2006). DNA-induced switch from independent to sequential dTTP hydrolysis in the bacteriophage T7 DNA helicase. *Mol. Cell,* **21**, 165–174.
35. Liao, J. C., Jeong, Y. J., Kim, D. E., Patel, S. S. & Oster, G. (2005). Mechanochemistry of T7 DNA helicase. *J. Mol. Biol.* **350**, 452–475.
36. Yu, X., VanLoock, M. S., Poplawski, A., Kelman, Z., Xiang, T., Tye, B. K. & Egelman, E. H. (2002). The *Methanobacterium thermoautotrophicum* MCM protein can form heptameric rings. *EMBO Rep.* **3**, 792–797.
37. Kusakabe, T., Baradaran, K., Lee, J. & Richardson, C. C. (1998). Roles of the helicase and primase domain of the gene 4 protein of bacteriophage T7 in accessing the primase recognition site. *EMBO J.* **17**, 1542–1552.
38. Mendelman, L. V., Notarnicola, S. M. & Richardson, C. C. (1992). Roles of bacteriophage T7 gene 4 proteins in providing primase and helicase functions *in vivo*. *Proc. Natl Acad. Sci. USA*, **89**, 10638–10642.
39. Lee, S. J. & Richardson, C. C. (2001). Essential lysine residues in the RNA polymerase domain of the gene 4 primase-helicase of bacteriophage T7. *J. Biol. Chem.* **276**, 49419–49426.
40. Ohi, M., Li, Y., Cheng, Y. & Walz, T. (2004). Negative staining and image classification-powerful tools in modern electron microscopy. *Biol. Proced. Online,* **6**, 23–34.
41. Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, M. & Leith, A. (1996). SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J. Struct. Biol.* **116**, 190–199.

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