Communication between subunits critical to DNA binding by hexameric helicase of bacteriophage T7

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The DNA helicase encoded by bacteriophage T7 consists of six identical subunits that form a ring through which the DNA passes. Binding of ssDNA is a prior step to translocation and unwinding of DNA by the helicase. Arg-493 is located at a conserved structural motif within the interior cavity of the helicase and plays an important role in DNA binding. Replacement of Arg-493 with lysine or histidine reduces the ability of the helicase to bind DNA, hydrolyze dTTP, and unwind dsDNA. In contrast, replacement of Arg-493 with glutamine abolishes these activities, suggesting that positive charge at the position is essential. Based on the crystallographic structure of the helicase, Asp-468 is in the range to form a hydrogen bonding with Arg-493 on the adjacent subunit. *In vivo* complementation results indicate that an interaction between Asp-468 and Arg-493 is critical for a functional helicase and those residues can be swapped without losing the helicase activity. This study suggests that hydrogen bonding between Arg-493 and Asp-468 from adjacent subunits is critical for DNA binding ability of the T7 hexameric helicase.

DNA replication | hydrogen bonding | subunit interaction | residue swappping

The replication, recombination, and repair of DNA all require the unwinding of duplex DNA. Such an important transformation of dsDNA into ssDNA is carried out by a group of proteins designated as DNA helicases. Underlying the overall process of unwinding dsDNA is the ability of the protein to bind to ssDNA and use the hydrolysis of a nucleoside triphosphate to translocate unidirectionally on the DNA (1, 2). Not surprisingly, DNA helicases differ in the mechanism by which they bind to DNA, translocate on ssDNA, and unwind the duplex. On the basis of their amino acid sequences and 3D structures, DNA helicases can be divided into several families or superfamilies (2, 3).

The helicase encoded by gene 4 of bacteriophage T7 is one of the most extensively characterized helicases. As a member of the DnaB-like family (family 4), the gene 4 protein forms a hexameric toroid. The oligomeric structure not only gives rise to the nucleotide bindings at the subunit interfaces but also provides a central cavity through which the ssDNA can pass. Binding sites for DNA and nucleotide span between subunits, thus allowing cooperative binding of DNA and efficient coupling of nucleotide hydrolysis to movement of the DNA strand.

Electron microscopy and x-ray crystallography illustrated that the protein forms a closed ring-shaped oligomeric structure composed of six or seven subunits (4–7). Biochemical studies have identified regions critical to oligomerization of the protein, including a linker region that connects the C-terminal helicase domain of the protein to the N-terminal primase domain (8, 9). Four motifs were defined based on conserved amino acid sequences among family 4 helicases. Strictly conserved residues in motifs 1 and 2 contact the nucleotide at the nucleotide binding site located at the interface of subunits. These residues participate in hydrolysis of nucleoside triphosphate (10, 11). Another conserved motif, motif 4, is located near the center of the hexamer, suggesting a role in DNA binding (Fig. 1). Indeed, mutagenesis studies supported such a role (12, 13). Upon binding

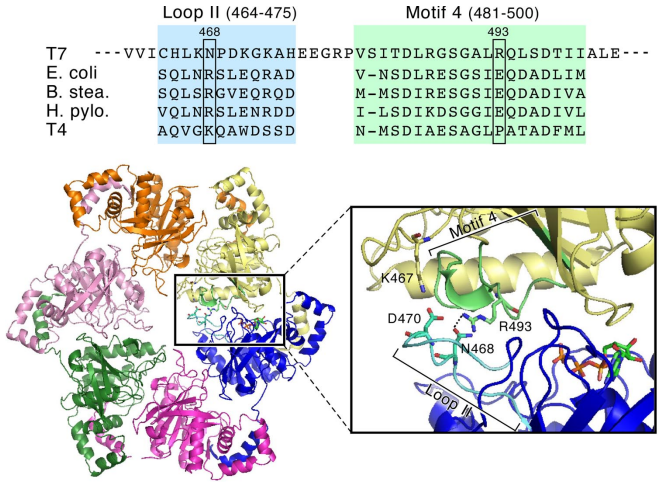

Fig. 1. Motif 4 within the central core of the hexameric gene 4 helicase. Amino acid sequences of loop II and motif 4 from several DnaB-like family helicases (T7 phage, *E. coli*, *Bacillus stearothermophilus*, *Helicobacter pylori*, and bacteriophage T4) are aligned (5). The crystal structure of the hexameric gene 4 protein (6) reveals a ring-shaped molecule with a central core through which the ssDNA passes. The individual subunits are identified by different colors. Within the central core, motif 4 (residues 481–500, colored in lime) of one subunit (shown in yellow) lies in close proximity to the DNA binding loop II (residues 464–475, colored in cyan) of the adjacent subunit (shown in blue). An ATP analog bound at the interface of subunits is shown in green and orange. Located within motif 4, R493 is in the proximity of two residues, N468 and D470, in the DNA binding loop of the adjacent subunit (*Inset*).

of the nucleotide, motif 4 assumes a helical conformation, suggesting a role for this motif in coupling NTP binding to DNA binding (6). In addition, loop II that protrudes inside the central hole of the hexamer also plays a role in DNA binding (14). Motif 4 from one subunit contacts loop II from an adjacent subunit at the interface of hexameric helicase.

The oligomeric state of the gene 4 protein is obviously essential for its helicase activity. The stability of the hexameric structure arises from two interactions. The first involves the interaction between adjacent subunits via an α-helix, and the second includes the interaction of residues within the nucleotide binding site located at the subunit interface. In the first, the N-terminal helix (residues 264–284) of each helicase subunit packs against the adjacent subunit to complete a four-helix bundle (residues 364–395) (6). Residues preceding this N-terminal helix are also essential for oligomerization and are parts of the linker region (residues 246–271) connecting the helicase

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and primase domains of the protein. The second interaction involving the nucleotide binding site is probably less important in maintaining stability but rather plays a more important role in sensing the state of hydrolysis of the nucleotide occupying this site. The presence of a nucleotide in the site does, however, increase the stability of the oligomer (15).

The mechanism by which the energy of hydrolysis of the nucleoside triphosphate is converted into unidirectional movement of the hexamer on ssDNA is slowly being elucidated. The crystal structure of the gene 4 helicase in the absence of DNA revealed subunits in three different states: empty, loosely bound NTP, and tightly bound NTP. The sequence of these states around the hexamer led Singleton *et al.* (6) to propose a sequential pathway in which all subunits participated in dTTP hydrolysis. Subsequent biochemical studies have largely validated this model (14, 16). A structure of the bovine papillomavirus (BPV) E1 helicase, a member of the AAA+ family, bound to an oligonucleotide and ADP, has provided insight into the actual coupling of nucleotide hydrolysis to translocation on ssDNA (17). The structure shows that the DNA binding loops protruding into the central core form a spiral staircase that sequentially tracks the phosphate backbone of the ssDNA. The height of each hairpin loop can be correlated with the particular nucleotide occupancy. In the resulting model, as the hydrolysis of NTP progresses through its catalytic cycle, the loops each move one step downward, thus pulling the ssDNA through the central core. NTP hydrolysis is in turn coordinated between adjacent subunits by specific interactions.

Extensive interactions of the gene 4 helicase with ssDNA occur within the central cavity formed by the six subunits. These interactions serve to move the DNA unidirectionally through the central core via conformational changes in the DNA binding loops. These conformational changes are fueled by the energy provided by hydrolysis of the nucleoside triphosphate located in the nucleotide binding site between the subunit interfaces, a considerable distance from the central core. Consequently, communication between these two sites must occur to coordinate DNA movement and nucleotide hydrolysis as the DNA is transferred from one subunit to another (6, 14, 16, 17). Motif 4 (residues 481–500) is located between these two crucial sites for helicase function (Fig. 1). This motif, conserved among this family of helicases, is rich in aliphatic residues but it also contains several charged residues (18). One of these charged residues was of particular interest because only T7 helicase has a positively charged arginine residue at the position of 493, whereas most of its homologs contain negatively charged glutamic acid (5). Consequently, we investigated the role of residue 493 by generating altered gene 4 helicases containing a single amino acid substitution.

Results

***In Vivo* Analysis of Alteration in Motif 4 of T7 DNA Helicase.** To determine the importance of residue R493 within motif 4 of the T7 gene 4 helicase, we replaced this residue with a variety of other amino acids. Table 1 summarizes all of the substitutions for R493 used in the present study and their ability to complement for the growth of T7 phage lacking gene 4. In this complementation assay, the viability of the phage lacking gene 4 depends on a functional gene 4 protein expressed from a plasmid harbored in *Escherichia coli*. Replacement of R493 with lysine reduced slightly the ability to support growth of T7 gene 4-deletion phage. In contrast, substitution of any other residue for R493 resulted in loss of complementation. The results clearly indicate that a long basic side chain at position 493 is required for the function of T7 gene 4 protein. None of the altered proteins exhibited a dominant negative effect on infection with WT T7 phage (data not shown). To identify defects resulting from the amino acid substitution, we purified and characterized biochemically three of the altered gene 4 proteins: gene 4 protein with R493 replaced with lysine (gp4-R493K), histidine (gp4-R493H), or glutamine (gp4-R493Q).

Table 1. Plating efficiency of T7Δ4 phage on *E. coli* strain containing plasmid expressing WT or altered gene 4 protein

Amino acid substitution	Efficiency of plating
WT	1.0
R493K	0.5
R493H	$<10^{-5}$
R493Q	$<10^{-5}$
R493N	$<10^{-5}$
R493D	$<10^{-5}$
R493E	$<10^{-5}$

Recombinant proteins containing the indicated amino acid substitutions were expressed in *E. coli* DH5. After infection with the indicated T7 phage, the number of plaques was counted and normalized to the value obtained with WT gene 4. Data were obtained from at least duplicate experiments.

Oligomerization. Functional gene 4 protein forms oligomers that can be detected by native PAGE. Comparisons of altered proteins with WT protein indicate that all of the proteins form oligomers in both the absence and presence of ssDNA and in amounts comparable to that observed with the WT gene 4 protein [supporting information (SI) Fig. S1].

Affinity for ssDNA. For gene 4 protein to translocate on ssDNA or unwind dsDNA, the protein must first bind ssDNA. DNA biding is conveniently measured by using a filter binding assay in the presence of β,γ-methylene dTTP. A functional gene 4 protein forms a stable DNA–protein complex in the presence of the nonhydrolyzable analog of dTTP; the complex can bind to nitrocellulose membrane whereas unbound DNA flows through. This filter binding assay shows that altered proteins in which R493 is replaced by lysine or histidine (gp4-R493K or R493H) have a lower affinity for ssDNA (Fig. 2). The binding affinity for gp4-R493K and gp4-R493H was decreased by 2- and 3-fold, respectively. Gp4-R493K and gp4-R493H also have lower maximum binding (80% and 48% of WT, respectively). On the other hand, substitution of glutamine for R493 (gp4-R493Q) eliminates all binding to ssDNA (Fig. 2).

dTTP Hydrolysis. Gene 4 protein hydrolyzes dTTP at a slow rate in the absence of ssDNA (12). However, upon binding to ssDNA,

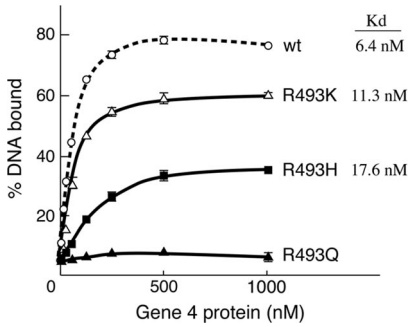

Fig. 2. ssDNA binding affinity of T7 gene 4 protein. The ability of gene 4 proteins to bind ssDNA was measured by filter binding assay. Radiolabeled 15-mer DNA (1 nM) was incubated with increasing amounts of gene 4 protein in the presence of 1 mM β,γ-methylene dTTP at 37°C for 10 min. The reaction mixture was passed through a nitrocellulose filter, and the DNA–protein complex bound to the filter was measured and plotted against the amount of protein. Dissociation constant (K_d) is indicated next to each gene 4 protein. Error bars are from two independent experiments.

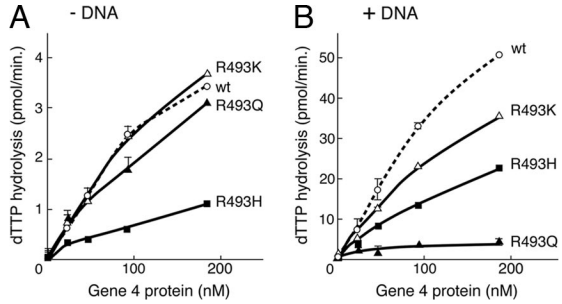

Fig. 3. dTTP hydrolysis activity of T7 gene 4 protein. (*A*) Increasing amounts of gene 4 protein were incubated with 100 μM dTTP (1 μCi [α-^{32}P] dTTP) at 37°C for 60 min in the absence of DNA. After separation of the reaction products by TLC, the amounts of hydrolyzed products were determined and plotted against the amount of protein. Error bars are from two independent experiments. (*B*) Assay similar to *A* was carried out in the presence of 6 nM M13 ssDNA. Reactions contained 250 μM dTTP (0.5 μCi [α-^{32}P] dTTP) and were incubated at 37°C for 20 min. Error bars are from two independent experiments.

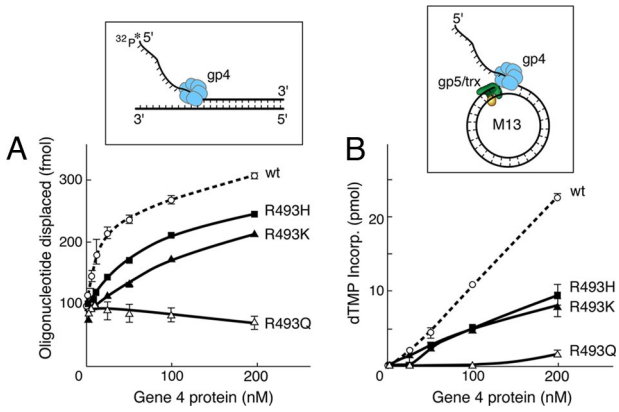

Fig. 4. Helicase activity of T7 gene 4 protein. (*A*) DNA unwinding activity of T7 gene 4 protein. The ability of gene 4 protein to unwind dsDNA was determined by using a minireplication fork consisting of two partially annealed DNA oligomers (see *Inset*). After incubation of 100 nM substrate at 37°C for 5 min in the presence of 1 mM dTTP and the indicated amounts of gene 4 protein, the reaction products were analyzed on 10% native gel and the amount of ssDNA unwound from dsDNA was measured. Error bars are from two independent experiments. (*B*) Strand-displacement DNA synthesis. The reaction contained 10 nM M13 dsDNA with a 5′-protruding tail (see *Inset*), 0.3 mM dNTP (0.5 μCi [α-^{32}P] dTTP), 20 nM T7 DNA polymerase, and an increasing amount of gene 4 protein. After incubation at 37°C for 20 min, incorporation of radioactive dTMP into DNA was measured. Error bars are from two independent experiments.

the rate of hydrolysis increases 50- to 100-fold. As discussed above, the energy derived from the hydrolysis of dTTP is used to fuel the translocation of the protein on ssDNA and unwind duplex DNA. In the absence of ssDNA, gp4-R493K and gp4-R493Q hydrolyzed dTTP at the same rate (3.4 μM/min per μM protein) as did the WT protein. Although gp4-R493H hydrolyzed dTTP at one-third the rate of WT gene 4 protein, the rate of hydrolysis depends on the amount of the protein, suggesting that this protein retains an active site for dTTP hydrolysis (Fig. 3*A*).

In the presence of ssDNA where the rate of hydrolysis of dTTP is greatly increased, gp4-R493Q displays only the same amount of hydrolysis as seen in the absence of ssDNA (Fig. 3*B*). Gp4-R493K and gp4-R493H hydrolyzed dTTP but at a considerably slower rate than that observed with WT gene 4 protein, 70% and 42%, respectively. The decrease is roughly proportional to the reduced affinity for ssDNA presented above.

DNA Unwinding. A major activity of gene 4 helicase during replication is unwinding dsDNA to create a ssDNA template for T7 DNA polymerase. We have examined the ability of gene 4 protein to unwind dsDNA by using a minireplication fork substrate (see Fig. 4*A Inset*). Not surprisingly, in view of the above results, gp4-R493Q is devoid of unwinding activity, whereas gp4-R493K and gp4-R493H have reduced unwinding activity compared with the WT protein (Fig. 4*A*).

Recent studies suggest that DNA synthesis catalyzed by T7 DNA polymerase assists the unwinding of activity of gene 4 helicase by destabilizing the junction between dsDNA and ssDNA at a replication fork (19). Consequently, a helicase defective in unwinding a duplex by itself might be assisted by concurrent DNA synthesis catalyzed by DNA polymerase. In the experiment presented in Fig. 4*B*, the ability of WT and altered gene 4 helicases to mediate leading strand DNA synthesis together with T7 DNA polymerase was measured (see Fig. 4*B Inset*). The reduced rates of leading strand DNA synthesis mediated by the altered helicases suggest that the lack of unwinding activity of the altered proteins cannot be rescued by the polymerase.

DNA Primase. Because R493 resides within the C-terminal helicase domain of gene 4 protein, primase activity located in the N-terminal domain is most likely not affected by mutations in this residue. In the experiment presented in Fig. 5, we have measured the ability of all three gene 4 proteins with substitutions for R493 to catalyze the synthesis of a functional tetraribonucleotide (pppACCC) on ssDNA containing the primase recognition sequence 5′-GGGTC-3′. Primase activity is enhanced greatly by the ability of the helicase to bind to ssDNA and thus increase the efficiency of sequence recognition by the primase domain (20). Therefore, to circumvent the influence of the defective DNA binding exhibited by these altered gene 4 proteins, we have used a short oligonucleotide (5′-GGGTCAAAAAAAAAA-3′) containing a primase recognition site; helicase binding to an oligonucleotide of this length is not significant (21). Indeed, all three of the gene 4 proteins with substitutions for R493 catalyzed the synthesis of tetraribonucleotides (Fig. 5). Within the experimental error of this assay, the

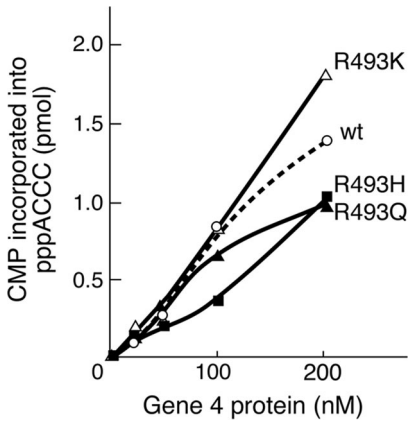

Fig. 5. Primase activity of T7 gene 4 protein. The ability of gene 4 protein to catalyze the synthesis of oligoribonucleotides was determined by using a 15-mer oligonucleotide containing a primase recognition site (5′-GGGTC-3′) as a template. The 15-mer (10 μM) was incubated with increasing amounts of gene 4 protein in the presence of 100 μM each of ATP and CTP (0.5 μCi [α-^{32}P] CTP) at 37°C for 20 min. Reaction products were analyzed on 25% polyacrylamide gel containing 3 M urea and the amount of CMP incorporated into product pppACCC was measured. Representative data from multiple experiments are presented.

Table 2. Plating efficiency of T7Δ 4 phage on *E. coli* strain containing plasmid expressing WT or altered gene 4 protein

Amino acid substitution	Efficiency of plating
Single substitution	
WT	1.0
N468R	$<10^{-5}$
D470R	$<10^{-5}$
D470K	$<10^{-5}$
Double substitution	
N468R / R493D	$<10^{-5}$
N468R / R493E	1×10^{-3}*
N468R / R493N	1.0
N468R / R493Q	$<10^{-5}$
D470R / R493D	$<10^{-5}$
D470R / R493E	$<10^{-5}$
K467D / D470K	$<10^{-5}$
Double expression†	
N468R + R493D	$<10^{-5}$
N468R + R493N	0.2

Recombinant proteins containing the indicated amino acid substitution were expressed in *E. coli* DH5. After infection with the indicated T7 phage, the number of plaques were counted and normalized to the value obtained with wild-type gene 4. Data were obtained from at least duplicate experiments.
*Small size of plaque.
†To avoid recombination between plasmids, plate was incubated for no more than 4 hr at 37°C.

rate and quantity of primer synthesis was comparable to that observed with WT gene 4 protein.

Swapping of Residue R493 and N468 Restores Complementation. Inspection of the crystallographic structure of the hexameric gene 4 protein reveals that there are two amino acid residues in close proximity to R493 (6). Functional groups from side chains of N468 and D470 on one subunit are in the range (2.5–3.3 Å) to form hydrogen bonds with the positively charged guanidino group of R493 in the neighboring subunit (Fig. 1). We speculated that the inability of the gene 4 protein having substitution of R493 to bind to ssDNA resulted from the loss of a critical interaction between R493 and either N468 or D470. Such a loss would arise because of a loss of the hydrogen bonding donor in the side chain of R493. To examine this possibility, we have made substitutions for N468 and D470 to evaluate their importance *in vivo* and identify substitutions that would restore defective complementation caused by substitutions for R493.

Both N468 and D470 are important as illustrated by the lack of complementation of T7Δ4, lacking gene 4, by gp4-N468R, gp4-D470R, and gp4-D470K (Table 2). All of these substitutions eliminate the hydrogen bonding acceptor at side chains. To determine whether alterations of residues at positions 468 and 470 could rescue gp4-R493D, R493E, R493N, or R493Q, we constructed plasmids harboring gene 4 with substitutions not only in R493 but also in either N468 or D470 (Table 2). Only one altered gene 4 (N468R/R493N) supports the growth of T7Δ4 as efficiently as does the WT gene 4. In this altered protein, the arginine and asparagine at positions 493 and 468, respectively, are swapped. Thus, the maintenance of this amino acid pair allows for complementation of T7Δ4 regardless of their locations at these two positions on gene 4 protein. The double substitution (N468R/R493E) does allow for growth of T7Δ4 but with an efficiency that is 1,000-fold lower than that obtained with WT gene 4. In addition, the sizes of the plaques are very small.

A critical interaction between R493 and N468 suggests an intermolecular interaction between neighboring subunits. To examine this hypothesis, we simultaneously expressed both gp4-N468R and gp4-R493N, two noncomplementing proteins in

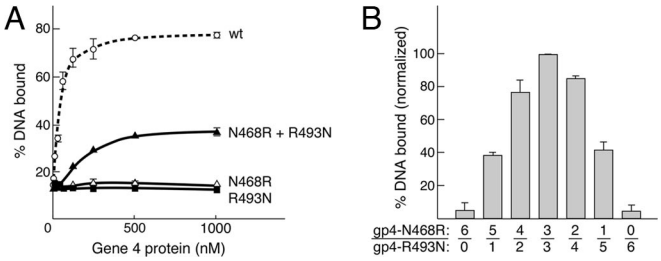

Fig. 6. Binding of gp4-N468R and gp4-R493N to ssDNA. (*A*) The ability of gp4-N468R and gp4-R493N to bind ssDNA was measured as described in Fig. 2. Gp4-N468R and gp4-R493N were mixed at a ratio of 1:1. Error bars are from two independent experiments. (*B*) DNA binding affinity of gene 4 protein mixture at various ratios. Mixing ratio of gp4-N468R and gp4-R493N was varied from 1:5 to 5:1. Binding affinity was normalized to the maximum binding affinity obtained from mixture at the ratio of 3:3. Error bars are from three independent experiments.

E. coli and tested for growth of T7Δ4. The *in vivo* complementation assay indicates that functional heterohexameric gene 4 proteins are formed from the two defective proteins (gp4-N468R and gp4-R493N). The efficiency of complementation is 5-fold lower than that observed with homohexameric proteins such as WT gene 4 protein or the altered protein in which residue R493 and N468 are swapped (Table 2). Such a decrease in complementation is not surprising considering the multiple arrangements of subunits that could arise during assembly of the hexamer. Because plasmids expressing the defective proteins contain the same coding region except for the indicated substitutions, a WT gene 4 protein could be generated by the T7 phage recombination machinery upon infection by the phage (22). To rule out a possible misinterpretation caused by the recombination products, we used another pair of defective proteins (gp4-N468R and gp4-R493D) as a negative control. Under the same incubation condition (4 h at 37°C) after T7 phage infection, the negative control did not yield any plaque formation. However, small plaques were observed when the incubation period was extended, suggesting that functional WT gene 4 protein can eventually arise by *in vivo* recombination.

Heterohexamer Between gp4-R493N and gp4-N468R Subunits Binds DNA. To confirm *in vivo* reconstitution of complementation ability from noncomplementing proteins, we purified the two defective proteins (gp4-N468R and gp4-R493N) and measured their ability to bind ssDNA in the presence of β,γ-methylene dTTP. As expected, gp4-R493N in which the positive charge on residue R493 is lost does not bind to ssDNA (Fig. 6*A*). Gp4-N468R, which could not support the growth of T7 phage lacking gene 4, also does not bind to ssDNA. This defect most likely is the explanation for its loss of activity *in vivo*. Whereas neither protein can bind ssDNA, a mixture of the two binds ssDNA ≈50% as well as does WT gene 4 protein (Fig. 6*A*). To determine whether either residue plays a more important role in DNA binding than the other, we mixed the two proteins at different ratios and determined the affinity for ssDNA. As shown in Fig. 6*B*, an equimolar amount of the two altered proteins resulted in maximal binding to ssDNA.

Discussion

The relationship of NTP binding and ssDNA binding by the T7 DNA helicase is complicated. Not only does the binding of NTP increase the stability of hexamers (15), a prerequisite for DNA binding, but NTP binding also increases greatly the efficiency of binding of the hexamer to ssDNA (23). Thus, any alteration in the gene 4 protein that affects either NTP binding or oligomerization of the protein disturbs binding of the protein to ssDNA.

For example, removal of the linker region between the primase and helicase domains eliminates oligomerization and hence DNA binding (8). Likewise, alterations in the nucleotide binding site that affect the binding of dTTP but do not drastically alter oligomerization either decrease or increase binding to ssDNA (10, 11, 14). A more direct effect on DNA binding is mediated by several basic residues on a loop that protrudes into the central core of the hexameric helicase and is thought to make physical contact with the negatively charge phosphodiester backbone of the ssDNA (6). Alterations in the basic residues in loop II, for example, decrease or eliminate binding to ssDNA (14).

In addition to these parameters that affect binding to ssDNA, there must certainly be segments of the protein that mediate information in the nucleotide binding site to sites involved in oligomerization and binding to ssDNA. For example, amino acid changes within motif 4 (residues 481–500) affect both oligomerization and ssDNA binding (12, 13). Although a portion of motif 4 forms the central cavity, the rest of this motif is not directly involved in contacting the NTP or the ssDNA. It has been proposed from structural studies with other helicases that residues in the DNA binding loop II make contact with residues in the same loop from the adjacent subunit to create conformational changes that move the DNA through the central cavity (17). In the present study, we have shown that one residue located in motif 4 of one subunit interacts with a residue in loop II of the adjacent subunit, most likely a hydrogen bond based on the crystal structure.

The Interaction Between Motif 4 and Loop II. Structural and biochemical studies suggest that a conformational switch dependent on the state of NTP binding provides the driving force for translocation on ssDNA and the unwinding of dsDNA (6, 16). Depending on the nucleotide binding states, structural changes created within the NTP binding pocket at the subunit interface must propagate toward the central channel where DNA binding occurs. However, the NTP binding site is distant from the central channel for DNA binding site. An interface created by motif 4 from one subunit and loop II region of an adjacent subunit is located in the middle of these two crucial components for helicase function. One interaction that occurs at this interface, hydrogen bonding between N468 of one subunit and R493 of the adjacent, is likely involved in the relay of conformational information generated within the NTP binding pocket to the central channel so that ssDNA moves from one subunit to the adjacent one.

Our data consistently demonstrate that basic characteristics at the position of residue 493 are essential for ssDNA binding ability by the gene 4 protein. Replacement of R493 with lysine or histidine retains most of activities of the protein, albeit slightly reduced activity. Confirming the requirement of a positively charged side chain at the position, any replacements with nonbasic residues abrogate most activities. The most conserved amino acid substitution, R493K, resulted in the least reduction in gene 4 protein activity. Although gp4-R493H does hydrolyze dTTP and unwind dsDNA, it cannot complement growth of T7Δ4 phage. In general, gene 4 protein is abundantly expressed under our *in vivo* complementation assay condition. Thus, many proteins defective in biochemical assays often display complementation, but the opposite occurs in the case of gp4-R493H. We do not have an explanation at this time other than that this residue may play an important role in a yet unidentified function.

Salt Bridges/Hydrogen Bonds Between Subunits. x-ray crystallographic structures of several helicases have revealed that salt bridges can be formed between charged residues conserved in various regions (24). A recent study on a hexameric helicase, BPV protein E1 demonstrated that a staircase is formed between basic and acidic residues in DNA binding hairpins of the adjacent subunits and this configuration plays a critical role in DNA binding by escorting ssDNA within the inside channel (17). K506 of this protein is engaged in charge-mediated interactions with three residues (D504, R505, and K508) in a hairpin found in the adjacent subunit to generate the staircase. From comparison of amino acid sequence, they suggested that a pair of residues in T7 gene 4 helicase, K467 and D470, might play a role in installing a staircase similar to that observed with D504 and K506 in BPV E1 helicase. The crystal structure of gene 4 helicase also indicates they are indeed in the range of hydrogen boding (2.3–3.4 Å) (6). The interaction of D470 with K467 was examined by constructing a double mutation K467D/D470K in which the two residues are swapped with each other. Both the single substitution D470 K and the amino acid swap K467D/D470K failed to complement growth of the T7Δ4, suggesting that K467 and D470 do not interact in a way similar to the case of N468 and R493 (Tables 1 and 2). The inability of these altered gene 4 proteins to complement most likely arises from the alteration in D470K. Consistent with this argument, alteration of K467A alone does not lead to a significant loss of complementation (S. Mukherjee, D. J. Crampton, and C.C.R., unpublished data). A precise role of D470 is not known but it is likely to have another charge-mediated, possibly more complicated, interaction because it is located within the polar environment of the internal cavity. Inconstant alignment of sequence caused by the diverse nature in helicase structures may explain the difference in interacting residues in T7 gene 4 helicase (family 4) and BPV E1 helicase (superfamily 3). Nevertheless, it is noteworthy that both helicases use charge-medicated interactions between subunits to exhibit ssDNA binding ability. It is also interesting to observe that other helicases in family 4, based on amino acid sequence alignment, contain an arginine–glutamic acid pair corresponding to N468-R493 of T7 helicase (Fig. 1) (5).

Hexameric Versus Heptameric State upon DNA Binding. T7 gene 4 protein can exist as a hexamer or a heptamer (6, 7). Although none of those structures contain ssDNA, examination of positions of the two residues identified in this study provides a clue to oligomeric state of T7 helicase upon binding to ssDNA. In the hexameric structure, the carbonyl oxygen of N468 is in proximity for hydrogen bonding with the guanidyl group of R493 (6). Distances between the two groups are marginally affected by the addition of nonhydrolyzable ATP (2.5–4.0 Å in the absence of the analog versus 2.7–3.3 Å in the presence of the analog). In contrast, side chains of the two critical residues identified in this study are too distant (>7.2 Å) for potential interaction in the heptameric structure (7). Moreover, the distances are varied from 7.2 to 14.5 Å depending on interfaces, which reflects a great deal of asymmetry in the structure. Therefore, based on the locations of these two residues within the heptamer, they could not interact to form the configuration necessary for DNA binding. Indeed, heptamers do not bind ssDNA (25).

In summary, a pair of residues, N468 and R493, play a critical role in ssDNA binding by forming a hydrogen bond between adjacent subunits within the central channel of the T7 helicase. The interaction not only connects two adjacent subunits but also provides a way to relay conformational transition in the NTP binding pocket to the DNA binding site. We propose that it is a common architecture for all hexameric helicases to have such networking at the interface of subunits. Undoubtedly, the example demonstrated here is only one of multiple communications between subunits.

Materials and Methods

Materials. Oligonucleotides were obtained from Integrated DNA Technology. Restriction endonucleases, M13 mp18 ssDNA, and Deep Vent polymerase were from New England Biolabs. T4 polynucleotide kinase, T4 DNA ligase, and nucleotides were purchased from Amersham Bioscience. Agarose was from

USB. DNA purification kits were from Qiagen. DNA binding membranes were from Bio-Rad.

Construction of Plasmids, Site-Directed Mutagenesis, Protein Overproduction, and Purification. Site-directed mutations were introduced into a plasmid, pET24-gp4, containing T7 gene 4 following a standard procedure. Altered gene 4 proteins were overproduced and purified as described (26).

Phage Growth Complementation Assay. Ability of T7 phage to grow on *E. coli* was determined by spot or plaque assay. First, for determination of sensitive range of phage concentration, an aliquot of *E. coli* culture grown at log phase was mixed with soft agar and plated on LB plate containing proper antibiotics. Small amounts (1–2 μl) of serially diluted phage solution were spotted and grown at 37°C for 5–6 h or room temperature overnight. Once the concentration of phage stock that would produce a countable number of plaques was identified, 0.3 ml of *E. coli* culture grown at log phase was mixed with 0.1 ml of phage stock and plated on an LB plate. After incubation, the number of plaques per plate was determined.

Biochemical Assays. Most of the biochemical assays (ssDNA filter binding, dTTP hydrolysis, DNA unwinding, strand-displacement DNA synthesis, primer synthesis, and oligomerization) used in this study have been described (9, 26). All reactions were carried out in a buffer containing 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 50 mM potassium glutamate. Briefly, ssDNA filter binding assays were carried out in a reaction containing 1 nM 5′-^{32}P radiolabeled 5′-GGGTCAAAAAAAAAA-3′, 1 mM nonhydrolyzable β,γ-methylene dTTP, and various amounts of gene 4 protein. Reaction mixtures were incubated at 37°C for 10 min and then loaded onto two layers of membranes, a nitrocellulose membrane (0.45 μM) laid above a Zeta-Probe (Bio-Rad) membrane. After washing with the buffer three times, protein–DNA complex bound to the nitrocellulose membrane and free DNA bound to the Zeta-Probe membrane were measured with a Fuji/BAS 1000 Bioimaging analyzer. Dissociation constant for DNA binding was obtained from a binding curve fit by nonlinear regression analysis using a computer program Enzyme Kinetics (Trinity Software). Dissociation constant represents hexameric concentration of T7 gene 4 protein at which 50% of the protein binds to ssDNA.

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