The arginine finger of bacteriophage T7 gene 4 helicase: Role in energy coupling

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The DNA helicase encoded by gene 4 of bacteriophage T7 couples DNA unwinding to the hydrolysis of dTTP. The loss of coupling in the presence of orthovanadate (Vi) suggests that the γ-phosphate of dTTP plays an important role in this mechanism. The crystal structure of the hexameric helicase shows Arg-522, located at the subunit interface, positioned to interact with the γ-phosphate of bound nucleoside 5′ triphosphate. In this respect, it is analogous to arginine fingers found in other nucleotide-hydrolyzing enzymes. When Arg-522 is replaced with alanine (gp4-R522A) or lysine (gp4-R522K), the rate of dTTP hydrolysis is significantly decreased. dTTPase activity of the altered proteins is not inhibited by Vi, suggesting the loss of an interaction between Vi and gene 4 protein. gp4-R522A cannot unwind DNA, whereas gp4-R522K does so, proportionate to its dTTPase activity. However, gp4-R522K cannot stimulate T7 polymerase activity on double-stranded DNA. These findings support the involvement of the Arg-522 residue in the energy coupling mechanism.

In the process of DNA replication, a single-stranded DNA (ssDNA) template is required for the proper base pairing of the incoming nucleotides by DNA polymerase. The transient unwinding of duplex DNA during replication is mediated by DNA helicases, a class of enzymes that couple the energy of hydrolysis of an NTP to conformational changes of the protein for unidirectional movement along the DNA (1, 2).

The replisome of bacteriophage T7 consists of gene 5 protein (DNA polymerase), *Escherichia coli* thioredoxin (processivity factor), gene 2.5 protein (ssDNA-binding protein), and gene 4 protein (DNA helicase and DNA primase) (3). The helicase and primase activities of most organisms reside in separate but interacting proteins (4). By contrast, in the T7 replication system, these activities are located in a single polypeptide, the 63-kDa gene 4 protein. The helicase domain is located in the C-terminal half of the polypeptide and the primase domain in the N-terminal half. A 26-aa segment linking the two domains is essential for oligomerization (5).

The gene 4 protein, in the presence of dTTP, assembles as a hexamer on ssDNA (6, 7) with a polarity such that the N-terminal helicase and C-terminal primase domains face the 3′ and 5′ ends of the DNA strand (6). On ssDNA, the protein then translocates unidirectionally 5′ to 3′ by using the energy of hydrolysis of dTTP (8, 9). If the protein encounters duplex DNA during its translocation, it will unwind the DNA. The movement of the gene 4 protein can be inhibited by a nonhydrolyzable analog such as β,γ-methylene dTTP (10, 11).

The crystal structures of the helicase fragment of the gene 4 protein revealed that the catalytic core resembles that of the *E. coli* RecA protein and the F_1-ATPase (12, 13). The crystal structure of residues 241–566 revealed that Lys-318, Glu-343, and Arg-522 are proximal to the γ-phosphate of the bound adenosine 5′-[β,γ-imido]triphosphate (ref. 13 and Fig. 1). Lys-318 is part of the Walker A homology (14) and is important for helicase function (15, 16), whereas Glu-343 has been postulated to be the "catalytic base" analogous to Glu-181 of the mitochondrial F_1-ATPase (17). Arg-522, which contributes to the nucleotide-binding pocket from the adjacent subunit, is similar to the arginine finger first identified in GTPases (18), and subsequently identified in other NTP-hydrolyzing enzymes such as the F_1-ATPase (19).

An arginine finger is recognized as an arginine residue that coordinates the γ-phosphate of a bound nucleotide, with the arginine residue being located distal to the nucleotide-binding site. The arginine can reside on a distinct activator protein, as in the case of GAP-Ras (20, 21), an adjacent subunit of an oligomeric protein such as the F_1-ATPase (17), or a distinct domain within the protein itself (22). Arginine fingers contribute to NTP hydrolysis through stabilization of the transition state of the reaction and as a trigger for conformational changes after hydrolysis of dTTP (19, 23, 24). Arginine fingers have been mutated in proteins such as the monomeric DNA helicase PcrA (25), as well as the AAA^+ proteins RuvB (26) and SF3 (27). In each case, nucleotide hydrolysis and DNA unwinding were adversely affected. Furthermore, the role of the γ-phosphate of the bound nucleotide in the catalytic mechanism has been probed in many NTPases by using phosphate analogs. Orthovanadate (Vi) complexes with nucleoside 5′ diphosphate and behaves as an analog of an NTP. Consequently, Vi plus ADP mimics ATP as substrate for various ATPase proteins (28–30). The binding of Vi plus ADP to these proteins results in the inhibition of ATP hydrolysis.

To produce locomotion, dTTP hydrolysis must be linked to conformational change (12, 13, 31). In the present communication, we describe studies using the T7 replicative DNA helicase encoded by bacteriophage T7 to address the role of the postulated "arginine finger" in energy coupling. Based on our data using altered gene 4 helicases, we propose that Arg-522 acts as an arginine finger whereby stabilizing a dTTP hydrolysis transition state through interactions with the γ-phosphate and triggering conformational changes essential for translocation along ssDNA.

Materials and Methods

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. Each reaction contained 0.8 mM protein, 1 mM β,γ-methylene dTTP, 2.5 mM 45-mer oligonucleotide, 40 mM Tris·HCl (pH 7.5), 10 mM MgCl, 10 mM DTT, and 50 mM potassium glutamate. The nondenaturing gel was run at 4°C in 1× TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) for 6 h at 5 V/cm. After staining with Coomassie blue, oligomerization of gene 4 proteins was verified by protein bands of >63 kDa (32).

dTTPase Assay for Gene 4 Protein. Gene 4 protein catalyzes the ssDNA-dependent hydrolysis of dTTP, a reaction coupled to its translocation on ssDNA (10). The reaction (10 μl) contained 1.1 nM M13 ssDNA, dTTP at the concentration indicated in the text (0.1 μCi [α-^{32}P]dTTP; 1 Ci = 37 GBq), 40 mM Tris·HCl (pH

Abbreviations: ssDNA, single-stranded DNA; Vi, orthovanadate.

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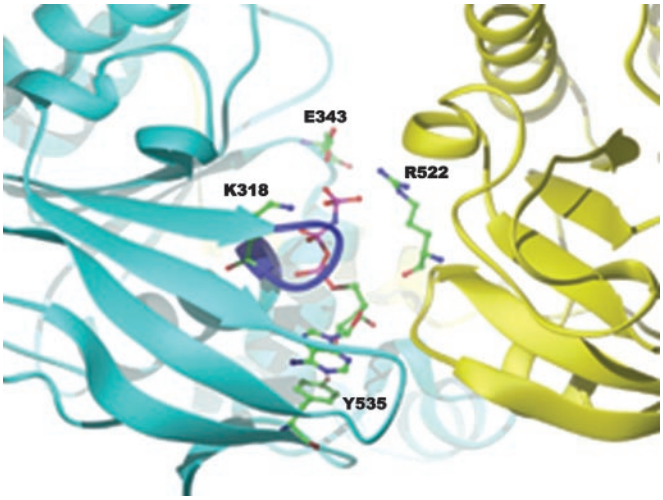

Fig. 1. The crystal structure of the interface between two subunits of T7 gene 4 helicase with adenosine 5′-[β,γ-imido]triphosphate in the nucleotide-binding site (13). Lys-318 of the P-loop (dark blue) and Glu-343 are within close proximity to the γ-phosphate of the bound nucleotide. Arg-522 from an adjacent subunit inserts into the nucleotide-binding site where it is in position to interact with the γ-phosphate of the bound nucleotide, an arrangement consistent with Arg-522 being an arginine finger.

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 5, 10, or 20 min, the reactions were terminated by adding EDTA to a final concentration of 25 mM. The reaction mixture was spotted onto a polyethyleneimine cellulose TLC plate and was 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 by 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 oligonucleotide (5′-A_{10}CTGGG-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 1 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 measured by using a Fuji BAS 1000 Bioimaging analyzer.

dTTP-Binding Assay. The dTTP binding affinity of gene 4 protein was measured by nitrocellulose filter binding as described in refs. 33 and 34 with minor changes. The assays were performed at 4°C by using a constant amount of protein and increasing concentrations of dTTP. Nitrocellulose filters (25 mm) were washed with 0.5 M NaOH for 10 min, were rinsed with double-distilled water, and were equilibrated in wash buffer (40 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM potassium glutamate). Gene 4 protein (24 μM) was mixed with dTTP (0–100 μM) and [α-^{32}P]dTTP in the absence of DNA in 40 mM Tris·HCl (pH 7.5)/10 mM $MgCl_2$/10 mM DTT/50 mM potassium glutamate/10% glycerol in a total volume of 20 μl. The reactions were incubated for 30 s on ice, and 10-μl aliquots were filtered through the nitrocellulose filter. The membranes were washed before filtration with 1 ml of ice-cold wash buffer and twice after filtration. The radioactivity (gene 4 protein–dTTP complex) bound to the nitrocellulose membrane was measured by using a Fuji BAS 1000 Bioimaging analyzer.

DNA-Unwinding Assay for Gene 4 Protein. A direct assay of helicase activity measures the release of a radioactively labeled oligonucleotide annealed to a complementary ssDNA. A helicase substrate was prepared by annealing 5′-^{32}P-labeled 45-mer oligonucleotide (5′-ATGACTCTATGCACATTGACATGCTTC AGATTCGTATTGTACACT-3′) to a 65-mer oligonucleotide (5′-T_{20}AGTCGTAATCCGACCTCGAGGCATTGTCAAT-GTGCATAGAGTCAT-3′) in 0.1 M NaCl. The reaction (10 μl) contained 200 nM DNA substrate, 4 mM dTTP, 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 50 mM potassium glutamate, and various amounts of gene 4 protein. After incubation for 5 min at 37°C, the reaction was terminated by the addition of EDTA to a final concentration of 25 mM, and then aliquots were loaded onto a 10% nondenaturing gel. The amount of radioactively labeled oligonucleotide separated from the partial duplex substrate by the gene 4 protein was measured using a Fuji BAS 1000 Bioimaging analyzer.

A trap for unwound substrate is not used to avoid decreasing the effective gene 4 protein concentration. In the absence of gene 4 protein, 50% of the 45 base and 65 base oligonucleotide anneal when incubated at 37°C for 5 min. As a result, the amount of unwinding observed in this assay is underestimated.

Gene 4 Protein-Mediated Strand Displacement Synthesis of T7 DNA Polymerase. Gene 4 protein stimulates strand displacement synthesis catalyzed by T7 DNA polymerase on duplex DNA (35). To monitor strand displacement synthesis, a circular duplex having a 5′ tail was used so that strand displacement synthesis could be performed by a rolling circle mechanism. The rolling circle DNA replication fork was created by priming M13 ssDNA with an oligonucleotide (5′-T_{36} AATTCGTAATCATGGTCATAGCT-GTTTCCT-3′) having 30 bases complementary to the M13 ssDNA and 36 bases forming a 5′ tail, then T7 DNA polymerase was used to completely replicate the M13 circle as described (36). Strand displacement synthesis was performed on the above double-stranded construct at 37°C in a reaction (100 μl) containing 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$,10 mM DTT, 50 mM potassium glutamate, 600 μM each of dATP, dCTP, dGTP, and dTTP, 0.1 μCi [α-^{32}P]dTTP, 10 nM double-stranded M13, 10 nM T7 DNA polymerase, and 40 nM gene 4 protein. After 5-, 10-, 20-, and 30-min incubations, EDTA was added to a final concentration of 25 mM to stop the reaction. Aliquots were placed on DE81 filter paper, were washed with 0.3 M ammonium formate (pH 8.0), and then the amount of radioactively labeled synthesized DNA bound to the DE81 filter was measured with a scintillation counter. DNA synthesis was monitored by the amount of [α-^{32}P]dTMP incorporated into the DNA.

Further information can be obtained in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Results

Vi Uncouples dTTPase and DNA-Unwinding Activities. Vi inhibits a number of nucleoside 5′ triphosphatases by forming a transition-state complex in the presence of Mg^{2+}-NDP. The formation of a Mg^{2+}-ADP·Vi transition-state-like complex has been demonstrated in the F_1-ATPase (30), the DNA-binding ABC ATPase MutS (37), and myosin (38). The structural similarities of the nucleotide-binding sites of the gene 4 helicase and other NTP-hydrolyzing enzymes suggests that Vi would be an inhibitor of gene 4 helicase.

Vi inhibits both the ssDNA-dependent dTTPase activity and the DNA-unwinding activity of T7 gene 4 helicase (Fig. 2). Because hydrolysis of dTTP by the gene 4 helicase is coupled to

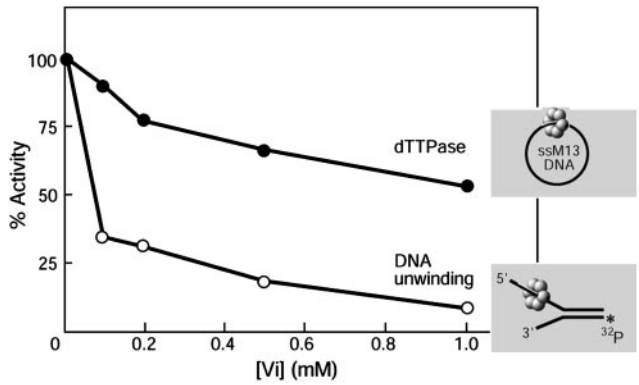

Fig. 2. Effect of Vi on dTTPase- and DNA-unwinding activities of T7 gene 4 helicase. Hydrolysis of dTTP was measured in a reaction containing 1.1 nM M13 ssDNA (see *Inset*), 1 mM dTTP, 0.1 μCi [α-^{32}P]dTTP, 80 nM of gene 4 protein, and the indicated amount of Vi. dTTPase activity (●) is expressed as a percentage of [α-^{32}P]dTTP hydrolyzed when the reaction is incubated at 37° for 20 min with respect to the value obtained in the absence of Vi. DNA unwinding was measured in a reaction containing 100 nM of a partial duplex of a 45-base oligonucleotide and 65-base oligonucleotide (see *Inset*), 80 nM gene 4 protein, 1 mM dTTP, and the indicated amount of Vi. Samples were incubated for 5 min and the reaction was stopped by the addition of EDTA. The products of the reaction were separated by electrophoresis by using a 10% TBE gel. DNA-unwinding activity (○) is expressed as a percentage of radioactively labeled 45-mer oligonucleotide separated from the partial duplex DNA substrate with respect to the value obtained in the absence of Vi. Each data point represents the mean of three experiments.

its translocation along ssDNA, a prerequisite for DNA-unwinding activity (11), it is not surprising that Vi also inhibits the unwinding of DNA. However, at 0.5 mM, Vi the unwinding activity is inhibited ≈75%, whereas the dTTPase activity is reduced by only 30% (Fig. 2). This difference in inhibition suggests that dTTP hydrolysis and DNA unwinding are not tightly coupled in the presence of Vi.

***In Vitro* Mutagenesis of Arg-522.** As an arginine residue that coordinates the γ-phosphate of bound nucleotide and originates from an adjacent subunit, Arg-522 has the appearance of an arginine finger. To explore this possibility, Arg-522 was substituted with an alanine (gp4-R522A) or a lysine (gp4-R522K) residue. T7Δ4-1, a phage in which the gene 4 coding region has been deleted, can grow only in *E. coli* cells harboring a plasmid that expresses a functional gene 4 protein (39). Plasmids expressing the two altered gene 4 proteins were transformed into *E. coli* C600. The host cells were then infected with T7Δ4-1. The plasmids containing substitutions of Arg-522 with alanine or lysine in the gene 4 protein did not support the growth of T7Δ4-1 (Table 1).

Table 1. Plating efficiencies of T7Δ4-1 and T7 wild-type phage on various *E. coli* C600 strains

Strain	T7Δ4-1* phage	T7 WT† phage
E. coli C600/pET11b	$<10^{-8}$§	1.2
E. coli C600/pET11b-63‡	1.0	1.0
E. coli C600/pET11b-63$_{R522A}$	$<10^{-8}$	0.85
E. coli C600/pET11b-63$_{R522K}$	$<10^{-8}$	0.90

*T7 phage lacking gene 4.
†Wild-type.
‡Plasmid expressing wild-type gene 4 protein.
§Efficiency of plating is calculated by dividing the number of plaque-forming units on a given strain by the plaque-forming units on *E. coli* C600/pET11b-63 (33).

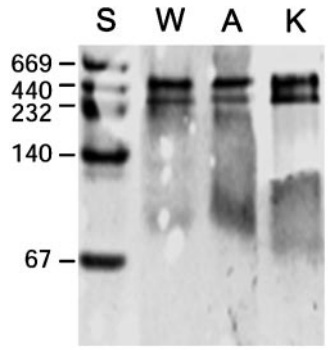

Fig. 3. Oligomerization of gene 4 proteins. The purified gene 4 helicases were examined for their ability to oligomerize by electrophoresis of the proteins under nondenaturing conditions by using a 10% polyacrylamide gel. Each lane contained ≈1.5 μg of the indicated gene 4 helicase and, after electrophoresis, the proteins were stained with Coomassie blue. W, wild-type; A, gp4-R522A; K, gp4-R522K; S, protein standard. The molecular masses (kDa) of marker proteins are shown on the left.

Protein Purification and Oligomerization. To analyze the role of Arg-522 biochemically, we purified the genetically altered gene 4 proteins, gp4-R522A and gp4-R522K. The altered proteins behaved similarly in the purification and the purity was >95% as judged by Coomassie blue staining of proteins after SDS/gel electrophoresis. Gene 4 protein oligomerizes to form a functional hexamer that binds to ssDNA (40, 41). As shown in Fig. 1, dTTP binds at the interface between pairs of adjacent subunits where Arg-522 lies close to the γ-phosphate of dTTP (12, 13). It was therefore critical to determine whether an alteration in Arg-522 affects oligomerization. As shown in Fig. 3, the genetically altered proteins form hexamers as does wild-type gene 4 protein.

Arg-522 Plays a Role in dTTP Hydrolysis. Given that the altered proteins form oligomers, we measured the ability of gp4-R522A and gp4-R522K to hydrolyze dTTP in the presence of ssDNA. Both gp4-R522A and gp4-R522K have significantly decreased dTTPase activities as compared with wild-type gene 4 protein (Fig. 4). The V_{max} values for gp4-R522A (1.7 pmol of dTTP hydrolyzed per s) and gp4-R522K (1.6 pmol of dTTP hydrolyzed

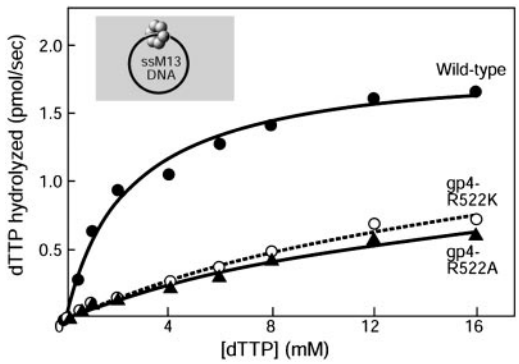

Fig. 4. dTTP hydrolysis catalyzed by gene 4 proteins. Gene 4 protein catalyzes the ssDNA-dependent hydrolysis of dTTP to dTDP and Pi. Each reaction contained 1.1 nM M13 ssDNA, the indicated concentration of dTTP (0.5, 1, 2, 4, 6, 8, 12, and 16 mM), 0.1 μCi of [α-^{32}P]dTTP, and 80 nM of the indicated gene 4 protein. At each dTTP concentration, the reactions were incubated at 37°C for 5, 10, and 20 min, then the product of the reaction ([α-^{32}P]dTDP) was analyzed by TLC as described in *Materials and Methods*. The rate of dTTP hydrolysis is plotted against the initial concentration of dTTP. Wild-type (●), gp4-R522A (▲), and gp4-R522K (○) are shown.

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Table 2. Comparison of basic properties of wild-type and altered gene 4 proteins

Activity*	Gene 4 proteins WT†	gp4-R522A	gp4-R522K
dTTP hydrolysis‡			
K_m, mM	3.3	22	18
V_{max}, pmol of dTTP hydrolyzed per s	2.2	1.7	1.6
dTTP binding§			
K_d, μM	12	11	19
DNA binding¶			
K_d, nM	18	8	6

*Results were obtained from multiple assays carried out as described in *Materials and Methods*. Kinetic constants were derived by using the program PRISM (GraphPad, San Diego).

†Wild-type.

‡K_m and V_{max} were derived from data as presented in Fig. 4.

§Dissociation constant for binding of dTTP to gene 4 protein as calculated from the retention of [α-^{32}P]dTTP-gene 4 protein on nitrocellulose at various concentrations of dTTP.

¶Dissociation constant for binding of hexameric gene 4 protein to ss DNA as calculated from the retention of ssDNA-gene 4 protein on nitrocellulose at various concentrations of gene 4 protein.

per s) are only slightly less than the wild-type rate (2.2 pmol of dTTP hydrolyzed per s). However, the K_m values for gp4-R522A (22 mM) and gp4-R522K (18 mM) are ≈6-fold greater than the K_m of wild-type gene 4 protein (3.3 mM, Table 2).

Because gene 4 protein is an ssDNA-dependent dTTPase (10), the low dTTPase activity observed with the altered proteins could be due to a lower affinity of the enzyme for either ssDNA or dTTP. The latter case is of interest because of the postulated interaction between the residue's guanido group and the γ-phosphate of dTTP (12, 13). Furthermore, the binding of gene 4 protein to ssDNA depends on the presence of nucleotide (42). However, the K_d values of the altered proteins for dTTP and DNA are similar to wild-type gene 4 protein (Table 2).

Effect of Vi on the dTTPase Activity of gp4-R522A and gp4-R522K. The residual dTTPase activities found in the altered proteins is unaffected by the presence of Vi (Fig. 5). The mechanism by which Vi inhibits NTP hydrolysis is through the mimicking of a transition state of the reaction. Thus, the lack of inhibition by Vi on the dTTPase activity of gp4-R522A and gp4-R522K suggests that the altered proteins have difficulty forming this particular dTTP hydrolysis transition state.

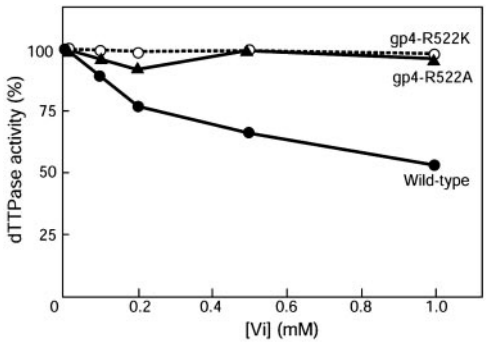

Fig. 5. Effect of Vi on the dTTPase activity of gp4-R522A and gp4-R522K. dTTPase activity is expressed as a percentage of dTTP hydrolyzed, with respect to the value obtained in the absence of Vi. Each reaction contained 1.1 nM M13 ssDNA, 1 mM dTTP, 0.1 μCi of [α-^{32}P]dTTP, 80 nM of the indicated gene 4 protein, and the indicated concentration of Vi. The reactions were incubated at 37° for 20 min and then the amount of [α-^{32}P]dTDP formed was analyzed by TLC, as described in *Materials and Methods*. Wild-type (●), gp4-R522A (▲), gp4-R522K (○) are shown. Each data point represents the mean of three experiments.

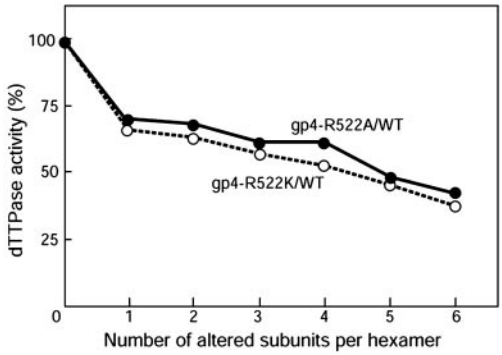

Fig. 6. dTTPase activity of heterohexamers composed of wild-type and variant gene 4 proteins. dTTPase activity is expressed as a percentage of dTTP hydrolyzed, with respect to the value obtained for wild-type gene 4 helicase. Each reaction contained 1.1 nM M13 ssDNA, 2 mM dTTP, 0.1 μCi of [α-^{32}P]dTTP, and 60 nM of the indicated mixture of gene 4 proteins. Before the reaction, wild-type gene 4 protein was mixed with gp4-R522A and gp4-R522K at the indicated ratio of subunits in the absence of dTTP at 37°C for 30 min. The reaction conditions are otherwise as presented in the legend to Fig. 5. Wild-type/gp4-R522A (●) and wild-type/gp4-R522K (○) are shown. Each data point represents the mean of three experiments.

Hetreohexamers of Wild-Type Gene 4 Protein and Altered Gene 4 Proteins. In view of the fact that the active state of gene 4 protein is oligomeric, we mixed wild-type and altered subunits (43) to examine dTTP hydrolysis catalyzed by heterohexamers. As shown in Fig. 6, even when there is on average only one altered subunit per hexamer, dTTPase activity is substantially decreased. Approximately 50% of the total loss of dTTPase activity observed in the altered homohexamer occurs when there is only one altered subunit per hexamer. Thus, one deficient subunit does not result in a 1 of 6 (17%) loss of dTTPase activity, but rather, a much larger loss (35%), suggesting that the presence of a single deficient subunit impaired dTTPase activity in the wild-type subunits as well. Comparable results have been reported for heterohexamers in which one of the subunits is deficient in dTTPase activity (15). This effect has lead to the conclusion that the subunits in the hexamer work in a cooperative manner.

Arg-522 Is Important for DNA Unwinding. Because the ability of the gene 4 protein to unwind DNA depends on dTTP hydrolysis, it is not surprising that the loss of Arg-522 leads to a deficiency in helicase activity (Fig. 7). The replacement of Arg-522 with an alanine (gp4-R522A) abolishes all helicase activity. However, gp4-R522K unwinds DNA ≈25%, as does wild-type gene 4 helicase. Although the altered proteins exhibit similar rates of dTTP hydrolysis, dTTPase- and DNA-unwinding activities are uncoupled in gp4-R522A, whereas gp4-R522K has maintained some coupling of the two activities. Even though the partial duplex DNA substrate used to measure DNA-unwinding activity underestimates the amount of DNA unwinding on a short DNA duplex region, the partial duplex DNA substrate does not measure extensive movement of the helicase along DNA. Hence, the observed helicase activities for wild-type and altered gene 4 helicases are not a good indication of helicase activity along extended stretches of duplex DNA.

Arg-522 Is Required for Helicase Stimulation of T7 DNA Polymerase on Duplex DNA. As shown in Fig. 8, in the absence of gene 4 helicase, T7 DNA polymerase catalyzes limited strand-displacement syn-

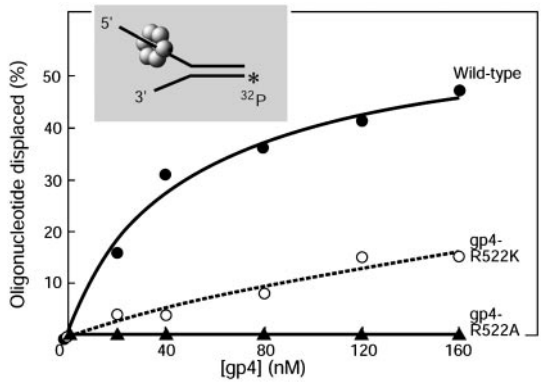

Fig. 7. DNA unwinding by wild-type and altered T7 gene 4 proteins. The helicase substrate consists of a 65-base oligonucleotide with 20 bases of a 45-base oligonucleotide annealed to its 3′ end as diagrammed in the *Inset*. The 45-mer was 5′-^{32}P-end-labeled so that its position in the gel can be detected and the amount released measured. The reactions contained 100 nM helicase substrate, the indicated concentration of gene 4 protein (12.5, 25, 50, and 100 nM), and 2 mM dTTP. Samples were incubated for 5 min and the reaction was stopped by the addition of EDTA. The products of the reaction were separated by electrophoresis, using a 10% TBE gel. The change in the amount of labeled 45-mer separated from partial duplex DNA substrate was calculated and expressed as a percent of total helicase substrate. Wild-type (●), gp4-R522A (▲), and gp4-R522K (○) are shown. Each data point represents the mean of three experiments.

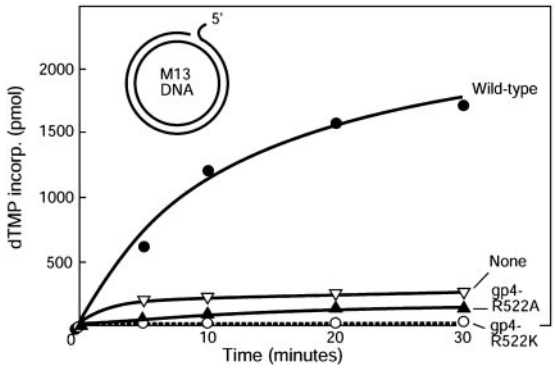

Fig. 8. Stimulation of T7 DNA polymerase by gene 4 proteins at a preformed replication fork. The ability of wild-type gene 4 protein (●), gp4-R522A (▲), and gp4-R522K (○) to stimulate T7 DNA polymerase on a DNA template containing a replication fork was measured. M13 dsDNA bearing a 5′ single-stranded tail (see *Inset*) was prepared as described in *Materials and Methods*. Each reaction contained 10 nM DNA, 600 μM each of dATP, dCTP, dGTP, and dTTP, 0.1 μCi of [α-^{32}P]dTTP, 10 nM T7 DNA polymerase, and 40 nM gene 4 protein. The amount of dTMP incorporated into DNA was determined at the indicated times of incubation. In a control experiment (▽), gene 4 protein was omitted from the reaction. Each data point represents the mean of three experiments.

thesis (44). However, in the presence of gene 4 helicase, T7 DNA polymerase catalyzes rapid and processive DNA synthesis on duplex DNA templates (45). Neither gp4-R522A nor gp4-R522K are capable of stimulating T7 DNA polymerase on duplex DNA. This result is not surprising, in view of their defects in nucleotide hydrolysis and DNA-unwinding activity.

Discussion

The insertion of Arg-522 into the nucleotide-binding site of an adjacent subunit (Fig. 1) closely resembles the arrangement of the arginine fingers that are present in the nucleotide-binding sites of G proteins such as Ras (20, 21) and the F_1-ATPase (17). In the case of G proteins, the arginine is provided by a GTPase-activating protein which, when bound, stimulates GTPase activity 10^5-fold (21). Likewise, the loss of the arginine finger in the hexameric proteins F_1-ATPase (19, 46) and termination factor ρ (47) leads to the loss of ATP hydrolysis.

The substitution of Arg-522 of T7 gene 4 protein with either an alanine or lysine gives rise to a helicase that cannot function *in vivo*. Gp4-R522A and gp4-R522K both hydrolyze dTTP 25% as does the wild-type gene 4 protein. Whereas alanine lacks the functional group necessary for interacting with the γ-phosphate, the amino group of a lysine may be able to approximate some of the specific interactions necessary for proper function of the enzyme. Consequently, gp4-R522A has lost the ability to unwind a short region of duplex DNA, whereas gp4-R522K exhibits reduced unwinding, roughly proportional to its dTTPase activity. Thus, the substitution of alanine for Arg-522 appears to have uncoupled dTTP hydrolysis and DNA unwinding, whereas the substitution with lysine results in the retention of some degree of coupling. The inability of Vi to inhibit dTTP hydrolysis catalyzed by the altered proteins suggests an interaction between Vi and the gene 4 protein is lost in the absence of Arg-522.

Interestingly, the residual DNA-unwinding ability of gp4-R522K is not adequate to support T7 DNA polymerase-catalyzed strand displacement synthesis. The inability of gp4-R522K to unwind DNA during DNA synthesis catalyzed by T7 DNA polymerase provides an explanation for the failure of gp4-R522K to support phage growth, even though it has DNA-unwinding activity. This result suggests gene 4 helicase must possess a minimal DNA-unwinding activity to sustain DNA synthesis catalyzed by T7 DNA polymerase.

In the crystal structure of the hexameric T7 gene 4 helicase, four of the six possible nucleotide-binding sites contained adenosine 5′-[β,γ-imido]triphosphate, whereas the remaining two sites appeared empty. This observation lead to the postulation of a binding change mechanism for NTP hydrolysis (13), whereby all six of the potential active sites are used at some point during multiple cycles of helicase activity. In this model, the binding and hydrolysis of nucleotides is sequential involving four NTP-binding sites at any one stage of the cycle, whereas the remaining two sites are unoccupied. In the sites that bind adenosine 5′-[β,γ-imido]triphosphate, Arg-522 is proximal to the γ-phosphate of the bound nucleotide, whereas in the empty sites it is displaced and cannot contribute to nucleotide binding. It was suggested that only those sites with Arg-522 near the γ-phosphate have sufficient affinity for nucleotide. Yet, the affinity of the gene 4 protein for dTTP is only slightly altered in the absence of Arg-522, suggesting the lack of nucleotide binding at the empty sites observed in the crystal structure is not simply explained.

The mechanism by which ssDNA-dependent dTTPase activity is uncoupled from helicase activity is most likely through the failure of dTTP hydrolysis to mediate conformational changes that lead to translocation on ssDNA (9, 13, 31). The β-sheet that begins at Arg-522 terminates at Tyr-535, a residue that coordinates the nucleotide base at the bottom of the metal-nucleotide-binding pocket (ref. 13 and Fig. 1). This arrangement provides a mechanism by which the subunits can monitor the nucleotide-binding state of adjacent subunits. The loss of coupling between dTTPase and helicase activities displayed by gp4-R522A and the preservation of that coupling by gp4-R522K suggests that helicase activity depends after transmission of conformational changes across subunit interfaces that rely on Arg-522. This proposition is supported by the observation that one subunit lacking Arg-522 per hexamer is sufficient to reduce dTTPase activity by ≈50%.

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