Nucleotide and DNA-induced Conformational Changes in the Bacteriophage T7 Gene 4 Protein*

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The bacteriophage T7 gene 4 protein is a multifunctional enzyme that has DNA helicase, primase, and deoxyribonucleotide 5’-triphosphatase activities. Prior studies have shown that in the presence of dTTP or dTDP the gene 4 protein assembles into a functionally active hexamer prior to binding to single-stranded DNA. In this study, we have examined the effects of different nucleotide cofactors on the conformation of the gene 4 protein in the presence and absence of DNA. Gel retardation analysis, partial protease digestion, and DNA footprinting suggest that the gene 4 protein undergoes a conformational change when dTTP is hydrolyzed to dTTP and that in the presence of dTDP the complex with DNA is more open or extended. We have also found that the dissociation constant of the gene 4 protein-DNA complex in the presence of dTDP was 10-fold lower than that determined in the presence of dTTP, further suggesting that these cofactors exert different allosteric effects on the DNA-binding site of the gene 4 protein.

The bacteriophage T7 gene 4 protein is crucial in the replication of the phage genome. In this process, it functions as a DNA helicase (Klodner et al., 1978; Klodner and Richardson, 1978; Matson et al., 1983) and primase (Strätling and Knippers, 1973; Hinkle and Richardson, 1975; Romano and Richardson, 1979a, 1979b). In addition, the gene 4 protein can hydrolyze deoxyribonucleotide triphosphates (dTTPs) to diphosphates, with dTTP being the preferred substrate (Matson and Richardson, 1983). The energy released from hydrolysis of dTTP is used to fuel the other biological activities of this enzyme, such as its unidirectional (from 5’ to 3’) translocation along single-stranded DNA (Tabor and Richardson, 1981) and its DNA unwinding activity.

Recently, it has been shown that the gene 4 protein is capable of forming a hexamer (Hingorani and Patel, 1993) that assembles in solution in the presence of either dTTP or dTDP, and in this form binds to single-stranded DNA. Following stabilization by cross-linking with gluteraldehyde, this hexameric complex can be isolated and was shown to retain both its DNA helicase and dTTPase activities implying that the functionally active form of the gene 4 protein is the hexamer. The hexameric structure formed by the gene 4 protein is capable of making simultaneous interactions with both strands of DNA, a complex in which two or three subunits are contacting the same strand.

Hydrolysis of dTTP by the gene 4 protein is required for both translocation along single-stranded DNA and unwinding of double-stranded DNA. However, little is known about how the gene 4 protein couples its dTTPase activity to the movement necessary for both of these biological processes. It is likely that a conformational change driven by the energy released from hydrolysis of dTTP may play a pivotal role producing the requisite movement that mediates the translocation and actual strand separation by the gene 4 protein. Indeed, nucleotide-induced conformational changes have been observed for several DNA helicases. These include Escherichia coli dna B protein (Nakayama et al., 1984) and E. coli Rep and Helicase II proteins (Chao and Lohman, 1990; Wong and Lohman, 1992). For the gene 4 protein, additional conformational changes might be induced by binding to single-stranded DNA since the dTTPase activity of the gene 4 protein is DNA-dependent (Matson and Richardson, 1983). It is possible that a structural alteration in the gene 4 protein triggered by the interaction with single-stranded DNA is necessary to initiate the hydrolysis of dTTP.

For these reasons it seemed that a knowledge of the allosteric effects on the structure of the gene 4 protein that are induced by nucleotide cofactors and the interaction with single-stranded DNA might help us understand how this enzyme couples the energy released by dTTP hydrolysis into molecular movement along the DNA strand. In the present study, we have examined the structural alternations in the gene 4 protein using gel retardation analysis, partial proteolysis, and nuclelease protection assays. The results from this study clearly show that the gene 4 protein undergoes conformational changes upon hydrolyzing dTTP to dTDP and upon binding to single-stranded DNA.

EXPERIMENTAL PROCEDURES

Materials

Enzymes—The T7 gene 4 protein was purified from extracts of E. coli 71.18 (obtained from Stan Tabor, Harvard Medical School) which harbored a plasmid over-expressing the wild type gene 4 protein. The expression of the cloned gene 4 was achieved by a T7 polymerase/promoter system (Tabor and Richardson, 1985). The gene 4 protein was isolated essentially as described (Bernstein and Richardson, 1988). All the gene 4 protein preparations used in the experiments were greater than 99% pure as judged by polyacrylamide gel electrophoresis in the presence of 0.1% SDS and staining the gel with silver. Trypsin was ordered from Sigma. Micrococcal nuclease was purchased from Amer sham Corp. The protein molecular weight markers were ordered from Boehringer Mannheim.

DNA and Nucleotides—Single-stranded M13mp9 DNA was purified as described (Messing, 1982). The homopolymers poly(dT) and poly(dA) and all the dinucleotides were ordered from Midland Certified Inc (Midland, TX). dTTP and dTDP were purchased from Pharmacia Biotech Inc. Commercially obtained dTTP contained typically 5–7% of contaminating dTTP and was purified by MonoQ iron-exchange high performance liquid chromatography to greater than 99.9% purity prior of DNA.1

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Methods

Gel Retardation Assays—The DNA-binding reactions (10 μl) were carried out in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, and up to 5% glycerol (from the enzyme stock). Each reaction typically contained 0.24 μM of the gene 4 protein, 0.24 μM of a 32P-labeled oligonucleotide, and 1 μM of either β,γ-methylene dTTP, dTDP, or dTDP as indicated. The reaction mixtures were incubated at 30 °C for 10 min, after which, 1 μl of a diluted glutaraldehyde (glutaric dialdehyde) solution was added to each reaction to a final concentration of 0.02%. The reactions were incubated for a further 20 s and then stopped by the addition of 6 μl of 100 mM EDTA, 40% glycerol, 0.1% bromphenol blue, and 0.1% xylene cyanol. The resulting mixtures were loaded onto a 6% non-denaturing polyacrylamide gel cast in 0.25 × Tris-borate-EDTA buffer. Electrophoresis was carried out at 12 V/cm and 4 °C with 0.25 × Tris-borate-EDTA as the running buffer. After electrophoresis, the polyacrylamide gel was subjected to autoradiography, and the radioactivity of the individual bands was quantitated by a phosphorimaging scanner (Molecular Dynamics SF) where indicated.

Preparation of Oligonucleotides with Different Sizes—In order to determine the minimum length of single-stranded oligonucleotide molecules that the gene 4 protein could bind under different conditions, we prepared a group of oligonucleotides ranging from 10 to 54 bases long with their length increasing by an increment of one base. Briefly, an oligonucleotide 54 bases long that had been labeled at the 5'-end with 32P was cleaved chemically as described for the A protein and oligonucleotides in the presence of dTDP and dTTP. The DNA-binding reactions and the separation of the reaction products were carried out as described under “Experimental Procedures.” An oligonucleotide molecule 60 bases long (60-mer) was used in this experiment. The compositions of these reactions are as indicated and the positions of the free 60-mer, and the protein-DNA complexes formed by the gene 4 protein and the 60-mer are indicated. The positions of the origins of the lanes are marked by the arrow.

RESULTS

The Gene 4 Protein-DNA Complexes Formed in the Presence of dTDP or dTTP

In the presence of either dTTP or dTDP, the gene 4 protein assembles into a hexameric complex in solution that binds to single-stranded DNA (Hingorani and Patel, 1993). The quaternary structure of this complex can be stabilized by the protein cross-linking agent glutaraldehyde, so that it remains stable during electrophoresis. A comparison of the electrophoretic mobility of the complex formed in the presence of dTDP and dTTP by gel retardation analysis is shown in Fig. 1. In the presence of dTDP, the gene 4 protein and a 60-mer formed a single protein-DNA complex that was detectable in the mobility-shift gel (Fig. 1). No change was observed if the gene 4 protein concentration was increased to 250 nM (Fig. 1). However, when dTTP was present, two differences were noted (Fig. 1). First, two shifted bands were observed that migrated substantially differently from each other in the gel. Second, the faster moving...
of these migrated slightly ahead of the complex formed when dTDP was used as the cofactor (Fig. 1). UV and gluteraldehyde cross-linking followed by SDS gel electrophoretic analysis of these faster moving complexes indicated that these materials contained a single gene 4 protein hexamer (data not shown). At higher concentrations of gene 4 protein in the presence of dTTP, only the slower moving of these two bands was observed suggesting that this complex is produced by the binding of two gene 4 protein hexamers to the oligonucleotide.

More interesting is the difference in mobility of the faster moving complexes formed in the presence of dTTP versus dTDP. The protein-DNA complex formed by the gene 4 protein and the 60-mer in the presence of dTTP displayed a clearly slower electrophoretic mobility than the complex formed at low (80 nM) concentrations of gene 4 protein in the presence of dTTP (Fig. 1). As described above, these complexes have identical molecular weights in an SDS gel, suggesting that the gene 4 protein-DNA complex may undergo a conformational change when the nucleotide cofactor is switch from dTTP to dTDP, a process that occurs during hydrolysis of dTTP. This observation also suggests that the gene 4 protein-DNA complex may adopt a more extended conformation in the presence of dTDP since this structure migrates more slowly in the gel than the complex formed in the presence of dTTP.

The Effects of Different Nucleotide Cofactors on the DNA Binding Activity of the Gene 4 Protein

Minimum Oligonucleotide Length—The formation of a stable gene 4-protein complex most likely requires the establishment of a minimum number of contacts between the DNA-binding site of the gene 4 protein and the single-stranded DNA. It is likely that the minimum length oligonucleotide that will bind to the gene 4 protein in the presence of either dTTP or dTDP is related to the conformation that the protein adopts: a more compact structure should bind to a shorter oligonucleotide while an extended structure should bind a longer one. To test this model, we generated a series of 32P-labeled oligonucleotides differing in length by one nucleotide and tested each for complex formation with the gene 4 protein.

In the presence of dTTP, the gene 4 protein could bind very weakly to an oligonucleotide as short as 12 bases in length (Fig. 2B). However, there appeared to be a sharp decrease in the formation of the protein-DNA complex when the oligonucleo-
tide probe was shorter than 13 bases long. As the length of the oligonucleotide probe used in the binding reaction increased, there was not only a general increase in the amount of the protein-DNA complex observed, but also the formation of a second complex with a lower electrophoretic mobility. In addition, the ratio of the second complex over the first increased steadily as the length of the oligonucleotide probe grew. This observation further substantiated the hypothesis that the slower-migrating complex was the result of contiguous binding of two gene 4 protein hexamers to a single oligonucleotide molecule.

When dTDP was present in the DNA-binding reaction as the nucleotide cofactor, the gene 4 protein could bind to a 21-mer efficiently; but when a 20-mer was used there was a dramatic decrease in the formation of the protein-DNA complex, and below this chain length no complex was observed (Fig. 2A). This suggested that in the presence of dTDP, an oligonucleotide of at least 21 bases long was required to provide sufficient interaction with the DNA-binding site of the gene 4 protein to obtain a stable protein-DNA complex. These observations also suggest that when the nucleotide cofactor associated with the gene 4 protein is switched from dTTP to dTDP, the allosteric effect induced by this transition caused structural perturbations that lead to an alternation in the DNA-binding site and presumably results in a more extended conformation since only longer oligonucleotides are bound.

Dissociation Constant of the Gene 4 Protein-DNA Complex—To gauge further the impact of different nucleotide cofactors on the DNA binding activity of the gene 4 protein, we examined the effects of dTTP and dTDP on the dissociation constants of the protein-DNA complexes formed by the gene 4 protein and single-stranded DNA. To avoid the possibility that two gene 4 complexes might bind to the oligonucleotide in the presence of dTTP, a situation which would complicate the data analysis (McGhee and von Hippel, 1974), we used a 25-mer probe for the analysis since only one hexamer complex bound to this length oligonucleotide at the protein concentrations used. Because dTDP induced only a single protein-DNA complex to form even with an oligonucleotide up to 60 bases in length (Fig. 2B) we used a longer oligonucleotide for the dTDP binding experiments to obtain higher binding efficiencies.

A fixed amount of the appropriate oligonucleotide probe was titrated with increasing concentrations of the gene 4 protein and the protein-DNA complexes formed were monitored by gel mobility-shift assay (Fig. 3A). In the presence of dTTP, the binding reaction reached a plateau when about 90% of the oligonucleotide probes were bound. However, when dTDP was present, the binding reaction reach a plateau after about 40% of the probes were associated with the gene 4 protein (Fig. 3A). These data were analyzed by a Hill plot (Fig. 3B) and the dissociation constants (K_d) calculated as described under "Methods". In the presence of dTTP, the K_d was 0.13 μM while dTDP gave a K_d of 1.3 μM. These results suggest the protein-DNA complex formed in the presence of dTTP was approximately 10 times more stable than the complex formed in the presence of dTDP. In fact, the difference in the K_d values reported here might well be underestimated because of the shorter length of the oligonucleotide molecule used in the presence of dTTP than in the presence of dTDP. In any case, these results are additional evidence that the transition from a dTTP-associating state to a dTDP-associating state has a major impact on the DNA-binding site of the gene 4 protein.

Partial Proteolytic Digestion Analysis of the Gene 4 Protein

Hydrolysis of dTTP to dTDP by the gene 4 protein occurs only in the presence of single-stranded DNA. Therefore, it seems likely that conformational changes in the gene 4 protein structure occur not only when dTTP is hydrolyzed to dTDP but also when the DNA binding takes place. To further investigate these putative conformational changes induced by nucleotide cofactors and single-stranded DNA, we conducted a series of limited proteolytic digestion analysis of the gene 4 protein. In these experiments, the gene 4 protein was preincubated with single-stranded M13 DNA, dTTP, or dTDP, or both DNA and the nucleotide cofactor, as indicated, and then trypsin was added and proteolysis was carried out for 10 min. Many studies have shown that whether a particular protease cleavage site is detectable is dependent on the conformation of the protein being digested. For example, this type of analysis has been used to provide information related to the conformation of a variety of protein substrates (Allan et al., 1992a, 1992b; Beekman et al., 1993; Bhat et al., 1993; Mark, 1994; Halazonetis et
Digestion of the gene 4 protein with trypsin produced a maximum of 14 discrete proteolytic fragments ranging from 21.2 to 53.8 kDa (Fig. 4). Because the amino acid sequence of the gene 4 protein contains 65 potential trypsin cleavage sites, it is possible that other proteolytic fragments also existed but were too short to detect in our electrophoretic system. Alterna-

**Fig. 4. Limited proteolysis of the gene 4 protein.** Proteolysis of the gene 4 protein with trypsin was carried out as described under “Experimental Procedures.” Lanes A and J in all panels are molecular weight markers; lanes I are trypsin. The compositions of each reaction are as indicated. The concentrations of trypsin present in lanes B through H are 0, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/μl, respectively. The positions of the undigested gene 4 protein molecules in all the panels are indicated by open arrows, and the positions of trypsin are indicated by closed arrows. The molecular weight of the markers are indicated on the left of each panel, the positions and the molecular weight of the proteolytic fragments are indicated on the right.
The products of tryptic digestion of the gene 4 protein under different conditions

The molecular weights of the proteolytic fragments of the gene 4 protein were determined by intrapolating their relative mobility into the molecular weight calibration curve generated with protein standards. The conditions under which tryptic digestion was carried out are indicated. ++, major tryptic cleavage products; +, minor proteolytic fragments.

| Proteolytic fragments | A | B | C | D | E | F |
|-----------------------|---|---|---|---|---|---|
| 53.8K | None | 270 μM | None dTDP | 270 μM | None dTTP | 270 μM |
| 48.2K | – | + | ++ | + | ++ | + |
| 45.9K | – | – | – | + | – | – |
| 44.4K | – | – | – | – | – | – |
| 39.7K | – | – | – | – | – | – |
| 38.1K | – | – | – | – | – | – |
| 35.5K | ++ | ++ | ++ | ++ | ++ | ++ |
| 34.1K | – | – | – | – | – | – |
| 32.2K | + | + | + | + | + | + |
| 26.4K | + | + | + | + | + | + |
| 25.1K | – | – | – | – | – | – |
| 23.8K | ++ | ++ | ++ | ++ | ++ | ++ |
| 22.6K | – | – | – | – | – | – |
| 21.2K | + | + | – | + | – | + |

* Panels shown in Fig. 4.

° ssM13 DNA.

Nucleotide.

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When neither single-stranded M13 DNA nor nucleotide cofactors were present, limited tryptic digestion revealed six proteolytic fragments (Fig. 4A and Table I). Addition of single-stranded DNA alone caused no detectable changes in the proteolytic pattern (Fig. 4B and Table I). This is consistent with the previous observations that nucleotide cofactors are absolutely required by the gene 4 protein in order to bind to single-stranded DNA (Matson and Richardson, 1985), and thus, in their absence, there is no interaction between the gene 4 protein and DNA and, therefore, no effect on the conformation of the gene 4 protein.

The addition of dTDP alone resulted in a few subtle differences in the digestion pattern (Fig. 4C). Under these condition, the 32.3- and the 21.2-kDa proteolytic fragments were not detected (Table I). Most of the other fragments were fainter than their counterparts in Fig. 4A and B, the exception being the 48.2-kDa fragment. In addition, the gene 4 protein was much more resistant to tryptic digestion in the presence of dTDP than when the reaction contained no nucleotides as shown by the level of gene 4 protein that remained when dTDP was present (cf. Fig. 4C with A and B). When the gene 4 protein was digested in the presence of both dTDP and single-stranded M13 DNA (Fig. 4D), the proteolytic pattern was drastically different from all those described above. At intermediate trypsin concentrations a total of 14 fragments were detected (Fig. 4D and Table I), some of which were not observed in the prior experiments. In addition, the gene 4 protein was more susceptible to the attack of the trypsin than when only dTDP was present. Taken together, these results suggest that the association with dTDP and the subsequent binding to single-stranded DNA triggered a major perturbation in the structure of the gene 4 protein and also that under these conditions the gene 4 protein was more susceptible to digestion, possibly because of a more open conformation.

When the gene 4 protein was preincubated in a reaction mixture containing dTTP but without single-stranded DNA, a total of five fragments were detectable (Fig. 4E and Table I). Subtle differences could be observed when this was compared with the experiment where only dTDP was present (Fig. 4C). First, the 35.5-kDa fragment was more resistant to tryptic digestion in the presence of dTTP. Second, we could observe the disappearance of the 26.4-kDa fragment and the appearance of a fragment 38.1 kDa in size. Third, the gene 4 protein appeared to be generally more resistant to the digestion of trypsin, as indicated by the relatively larger amount of the uncleaved gene 4 protein molecules left at the end of the protease digestion. Taken together, these results indicated that the gene 4 protein adopted a different conformation when it was bound to dTTP than when bound to dTDP.

Finally, limited proteolysis of the gene 4 protein was carried out in the presence of both dTTP and single-stranded M13 DNA (Fig. 4F). Under these conditions, a total of nine proteolytic fragments were generated. Compared with the profile where only dTTP was present (Fig. 4E), the proteolytic fragments, in general, started appearing at much lower concentrations of trypsin and with a much stronger intensity, suggesting that upon binding to single-stranded DNA, the gene 4 protein underwent structural alterations so that the trypsin cleavage sites involved were more accessible. When this profile was compared with that where both dTTP and single-stranded M13 DNA were present (compare Fig. 4F with Fig. 3D), the difference was even more drastic. In the presence of dTTP and single-stranded DNA (Fig. 3F), not only were the 53.8- and 45.9-, 44.4-, 34.1- and 22.6-kDa fragments missing, but the others like the 32.3-, 25.1- and the 23.8-kDa fragments were also much lower in their intensities and/or started appearing only at higher trypsin concentrations than in the presence of dTTP and DNA (Fig. 4D). In addition, in the presence of dTTP and DNA, the 35.5-kDa fragment appeared to be much more resistant to tryptic digestion than when dTDP was present, suggesting that the three-dimensional structure of this part of the gene 4 protein was different from the structure when dTDP was present. Finally, the dTTP-gene 4 protein-DNA complex was more resistant to tryptic digestion than the dTDP-gene 4 protein complex, as judged by the difference in the amount of the intact gene 4 protein molecules left after proteolysis.

The gene 4 protein hydrolyzes dTTP to dTDP in the presence of single-stranded DNA; therefore, after the gene 4 protein is incubated with dTTP and single-stranded M13 DNA, the nucleotide present would actually become a mixture of both dTTP and dTDP. Thus, it is possible that the protease digestion profile observed in the presence of dTTP resulted from the joint effects of dTTP and dTDP which were present in the ratio of 3:1 at the end of the protease digestion (data not shown). In order
to exclude this possibility, we used a nonhydrolyzable analog of dTTP, β,γ-methylene dTTP, which also induces strong binding of the gene 4 protein to single-stranded DNA. Limited tryptic digestion of this DNA complex gave results identical to that which were observed in the presence of dTTP (data not shown).

Overall, these results suggested that nucleotide cofactors triggered structural alterations in the gene 4 protein. The subsequent binding of the gene 4 protein to single-stranded DNA caused additional conformational changes that appeared to make it more open or accessible than in the absence of DNA. More importantly, the gene 4 protein adopted different conformations in the presence of dTTP versus dTDP and appeared to be more open when bound to dTDP.

Protection of Single-stranded DNA from Nuclease Digestion by the Gene 4 Protein

The previous results suggested that when the gene 4 protein bound to single-stranded DNA it assumed a more open conformation in the presence of dTDP than when dTTP was present. To confirm this supposition, we attempted to directly visualize these changes by examining the difference in the size of the single-stranded DNA fragments protected by the gene 4 protein in the presence of dTTP or dTDP. The size of the DNA fragments protected could be used as a "molecular ruler" to gauge the physical dimension of the gene 4 protein in its different conformational states. Micrococcal nuclease was used for these experiments because this endonuclease degrades single-stranded DNA in a relatively nonsequence-specific manner producing DNA fragments bearing a 5'-OH group. These fragments can be directly labeled with kinase under conditions where the nuclease is inactivated (addition of EGTA). For these experiments, we used dTDP and β,γ-methylene dTTP to prevent the gene 4 protein from translocating off the DNA fragments. Finally, the results obtained using homopolymers templates (poly(dT) and poly(dA)) are shown because native single-stranded viral DNAs produced anomalous results, possibly because of duplex secondary structures and/or the preference of micrococcal nuclease for AT-rich regions.

The gene 4 protein was preincubated with the appropriate nucleotide cofactors and poly(dT) molecules with an average length of 2000 bases, and then micrococcal nuclease was added. When dTDP was present, the ladder produced by the oligonucleotide fragments protected by the gene 4 protein centered at about 11–12 bases in length at low nuclease concentrations (Fig. 5A, lane F). As the amount of micrococcal nuclease used in the digestion increased, the fragments were further shortened to a lower limit of around five to six bases. It was possible that the 11–12-base long fragments reflected the physical dimension of each individual gene 4 protein molecule within the hexameric complex whereas the five to six-base fragments might represent the size of the DNA-binding site of the gene 4 protein.

The micrococcal nuclease digestion profile in the presence of dTTP was then determined. At low concentrations of micrococcal nuclease, the fragments protected by the gene 4 protein appeared to segregate into two groups; the ladder generated by the slower migrating group centered at about 21–22 bases while that produced by the faster migrating group centered at about seven to eight bases (Fig. 5A, lane J). As the concentration of the nuclease used in the digestion increased, the overall

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**FIG. 5.** DNA footprinting analysis of the gene 4 protein-DNA complex. The binding of the gene 4 protein to single-stranded homopolymers (poly(dT) or poly(dA)) and the subsequent nuclease digestion were carried out as described under "Experimental Procedures." The concentrations of micrococcal nuclease used in these reactions was 0.2 (lanes A and C), 0.5 (lanes B and D), 1.0 (lanes F and J), 2.0 (lanes G and K), 4.0 (lanes H and L), and 8.0 (lanes I and M) units/µl, respectively, for both panels A and B. Lanes E and N are size markers generated by combining the A, G, C, and T Sanger's dideoxy sequencing reaction mixtures. The primer-template complex used in the sequencing reaction contains a 5' end-labeled 11-mer annealed to a 48-mer. A, poly(dT); B, poly(dA).
pattern was similar, except that the average length of the upper ladder shrank to about 18–19 bases and that of the lower ladder declined to about four to five. One interpretation of these results (see "Discussion") is that the slower moving material represents protection by two adjacent gene 4 protein monomers while the faster bands again represent that within the DNA-binding site. This interpretation is consistent with our observation that two of the monomers simultaneously interact most strongly with the DNA.1

Taken together, these results indicate a marked difference in the profile of protection from nuclease digestion by the gene 4 protein in the presence of dTDP and dTTP. One interpretation of the data is consistent with the model that the gene 4 protein has a more open or extended conformation in the presence of dTDP. The results presented in this paper have important implications with regard to the mechanism of molecular movement by gene 4 protein. We find that dTDP and dTTP exert different allosteric effects on the gene 4 protein suggesting that the gene 4 protein alternates between these two different conformations during hydrolysis of dTTP. The differences in $K_d$ for these two complexes suggest that when dTDP is bound, the protein-DNA complex is at a higher energy state, and therefore the displacement of dTDP by dTTP in the nucleotide-binding site of the gene 4 protein is favored and would result in release of free energy. This, in turn, implies that the energy input used to drive the transition from a dTDP-induced conformation to a dTTP-induced one could potentially be provided by the free energy released during this process, while the energy required to effect the structural alternation in the opposite direction is supplied through hydrolysis of dTTP. It is likely that the hydrolysis of dTTP to dTDP and the subsequent displacement of dTDP by a dTTP molecule cycles the gene 4 protein through these different conformational changes and somehow results in unidirectional translocation and the helicase activity.

We have also shown that when the gene 4 protein binds to single-stranded DNA in the presence of dTDP, it adopts a conformation that is more open or extended and interacts with a longer stretch of DNA than the conformation formed in the presence of dTTP. During hydrolysis this conformation change occurs within the context of a hexameric complex and therefore would introduce structural constraints into the complex itself. It is thought that the gene 4 protein translocates along single-
stranded DNA in the 5' to 3' direction, raising the possibility that any conformational changes that bring about these movements are also directional. Thus, it is tantalizing to hypothesize that the sequential vectoried extension of the structure of these individual protomers causes the protein complex to move in a single orientation in a rolling type process (Lohman, 1993). Recently an EM study of the gene 4 protein hexamer bound to single-stranded DNA suggested that the DNA passes through the center of the ring monomeric structure (Egelman et al., 1995). Although our data do not exclude this possibility, an alternative model, in which the DNA contacts two or three monomers on the outside of the hexameric ring (Debyser et al., 1994) would allow a "rolling" mechanism for unwinding (Lohman, 1993), and is supported by several experiments. First, we find that micrococcal nuclease is able to digest all but five to six nucleotides, a fact that can be accommodated in the "outside binding" model if this is the size of the DNA occluded in the DNA-binding site. From the dimensions of the gene 4 protein hexamer as visualized in the EM, the alternative "inside binding" model predicts that each hexamer would protect approximately 25–30 nucleotides (Egelman et al., 1995). Second, it is well established that the gene 4 protein forms hexamers in the presence of nucleotide cofactor in the absence of DNA (Patel and Hingorani). In order for this complex to then bind to circular DNA by the inside binding model, one would need to postulate that the hexamer disassembles prior to DNA binding. Third, we and others (Egelman et al., 1995) have shown that protein-protein cross-links do not form a complex with single-stranded linear DNA that is stable to denaturation, although this complex apparently is stable if the DNA is circular (Egelman et al., 1995). It is possible that the single-stranded DNA slips out through the inside of the hexameric ring after denaturation; however this seems unlikely for DNA that is 7000 bases long and bound to multiple gene 4 protein complexes. Finally, UV cross-linking of the gene 4 hexamer to DNA showed very strong and simultaneous binding between two of the subunits and one DNA strand. The inside binding model not only predicts difficulty in forming a UV cross-link because of the inaccessibility of the DNA to the UV light, it also predicts a more symmetrical interaction between the two subunits and the DNA. Which of these two possible models is correct awaits further study.

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