Oligomeric Structure of Bacteriophage T7 DNA Primase/Helicase Proteins

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The oligomeric structure of bacteriophage T7 gene 4 helicase/primase proteins was investigated using protein cross-linking and high pressure gel-filtration chromatography. Studies were carried out with both 4A' and 4B proteins. 4A' is a M64L mutant of 4A which has similar helicase and primase activities as the wild-type mixture of 4A and 4B proteins (Patel, S. S., Rosenberg, A. H., Studier, F. W., and Johnson, K. A. (1992) J. Biol. Chem. 267, 15013–15021), and 4B is the smaller protein which has only helicase activity. Chemical cross-linking of 4A' and 4B proteins with dimethyl suberimidate resulted in cross-linked species ranging from dimers to hexamers and beyond. The cross-linking time course, however, indicated that hexamers were the predominant species to accumulate in both 4A' and 4B proteins. The effect of MgNTP and DNA binding on oligomerization of the gene 4 proteins was investigated using high pressure gel-filtration chromatography at increasing protein concentrations. In the absence of added ligands, close to 100 μM protein concentrations were required to form stable oligomers beyond dimers. However, in the presence of Mg-β,γ-methylene deoxythymidine triphosphate (nonhydrolyzable analog of dTTP), 4A' and 4B proteins assembled into stable hexamers at protein concentrations less than 8 μM. Addition of single-stranded DNA further stabilized the hexamer structure. Therefore, in the presence of a 60-nucleotide-long single-stranded DNA, hexamers were observed at protein concentrations as low as 0.2 μM. Nuclease protection experiments indicated that the 4A' and 4B hexamers protect about 60–65 bases of single-stranded DNA.

A number of biological processes, including DNA replication, DNA repair, recombination, translation, as well as transcription initiation and termination, require single-stranded DNA or RNA as substrates. These single-stranded substrates are generated when duplex DNA and RNA molecules are unwound by enzymes known as helicases. The enzyme-catalyzed unwinding process is facilitated by a DNA-dependent NTPase activity which is present in all helicases. A number of DNA helicases and several putative RNA helicases have been identified at the present time (Matson and Kaiser-Rogers, 1990 and Schmid and Linder, 1992). Only a few of these have been studied in any detail, and the mechanisms of DNA unwinding and NTP hydrolysis remain largely unknown. Interestingly, several helicases are now known to form oligomers (Lohman, 1992). Helicases seem to show a preference to self-assemble either as hexamers or dimers. For example, helicases such as Escherichia coli DnaB (Arai et al., 1981), SV40 and polyoma large T antigen proteins (Mastrangelo et al., 1989; Wang and Prives, 1991), and E. coli Rho protein (Finger and Richardson, 1982) form hexamers, whereas E. coli helicases, including Rep (Chao and Lohman, 1991), helicase II (Runyon et al., 1993) and helicase III (Yarranton et al., 1979), as well as HeLa helicase (See et al., 1991), form dimers. Exactly how oligomerization facilitates DNA unwinding is not understood. Mechanisms have been proposed for helicases such as E. coli Rho (Geiselmann et al., 1992) and E. coli Rep protein (Wong and Lohman, 1992) in which oligomerization has been suggested to play an important role in facilitating translocation and unwinding.

We are interested in understanding the mechanism of a DNA helicase protein from bacteriophage T7. The T7 DNA helicase is responsible for unwinding DNA ahead of the polymerase during DNA replication. The T7 proteins are model systems to study the complex mechanisms of DNA replication, because the bacteriophage encodes one of the simplest protein systems which is both economical and highly efficient in catalyzing DNA replication. The helicase activity of T7 is found both alone as the 4B protein and in association with the primase activity as the 4A protein (Bernstein and Richardson, 1988a, 1989). Both 4A and 4B proteins are products of the same gene, synthesized from the same mRNA with initiation sites that are 189 nucleotides apart (Dunn and Studier, 1983). 4A is thus the larger protein which is ~63 kDa in size and 4B is the smaller protein whose mass is ~56 kDa. Even though 4A contains both the helicase and primase activities, and 4A alone can support essentially normal replication and phage growth (Rosenberg et al., 1992), the 4B protein is made in approximately equal amount as the 4A protein during phage infection (Dunn and Studier, 1983).

Both 4A and 4B proteins have been overexpressed together and separately in E. coli. Protein purification from recombinant cells expressing 4A and 4B proteins results in a mixture of gene 4 proteins, because the proteins are difficult to separate due to their very similar properties (Nakai and Richardson, 1988). To study the biochemical properties of the individual protein, each protein was expressed separately. A clone for 4B alone was prepared by simply deleting the initiation codon of 4A (Bernstein and Richardson, 1988b; Rosenberg et al., 1992). To express 4A alone, however, it was necessary to shut down 4B synthesis. This was accomplished by Mendel- man and Richardson (1991) through synonymous codon replacement in the 4B ribosome-binding site. Mutation of the ribosome-binding site reduced 4B synthesis, hence 4A protein purified from this clone contained less than 0.5% contaminating 4B. Rosenberg et al. (1992), on the other hand, mutated the 4B initiation codon to express 4A alone. The 4B initiation codon for methionine was replaced by bases that code for

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purified separately from recombinant primase activities as the wild-type protein (Rosenberg et al., 1992; Patel et al., 1992). This was reported in this paper, along with 4A' and 4B proteins that were purified separately from recombinant E. coli cells.

Oligomerization of the gene 4 proteins has been suggested in the literature for some time (Nakai and Richardson, 1988; Bernstein and Richardson, 1988). However, only recently 4A' and 4B proteins were shown to form dimers and higher order oligomers by gel-filtration and native polyacrylamide gel electrophoresis (Patel et al., 1992). Because these studies were carried out at a single protein concentration and in the absence of MgNTP and DNA, it was not clear whether dimers were the intermediate species to formation of higher oligomers. In order to begin studies to understand the mechanism of the helicase/primase protein, it was important to first establish the native structure of the gene 4 proteins. We report here a detailed investigation of the oligomeric structure of the gene 4 proteins using protein cross-linking and small-zone gel-filtration chromatography. Oligomerization was studied as a function of increasing gene 4 protein concentrations both in the absence and in the presence of MgTP-PCP and DNA. Our results show that both 4A' and 4B proteins can form stable hexamers in the presence of MgTP-PCP and DNA.

**EXPERIMENTAL PROCEDURES**

**Reagents and Buffers**—The following reagents were purchased commercially and used without further purification. dTTP was purchased from Pharmacia LKB Biotechnology Inc. dTMP-PCP was purchased from United States Biochemical Corp., and DMS and TEA were purchased from Sigma. Radiolabeled compounds [α-32P]TTP (3000 Ci/mM) and [γ-32P]ATP (4000 Ci/mM) were purchased from ICN Radiochemicals.

All buffer solutions were prepared from reagent-grade chemicals. Chemical cross-linking experiments were performed in 50 mM TRIS-HCl, pH 8.2, 100 mM NaCl, 10 mM MgCl2, 0.1 mM EDTA, and 1 mM DTT. High-pressure gel-filtration elution buffers contained 50 mM Tris-Cl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, and when dTMP-PCP was present, 10 mM MgCl2 was added. The nuclease protection experiments were conducted in buffer containing 20 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 2.5 mM CaCl2, 1 mM DTT, and 50 mM NaCl.

Proteins and Enzymes—4A' and 4B proteins were purified to >95% purity from clones pAR5018/HMS174(DE3) and pAR3708/HMS174(DE3), respectively, as described earlier (Patel et al., 1992). Protein concentrations were determined spectrophotometrically with absorbance measurements at 280 nm and using the extinction coefficients of 83,600 and 69,000 M⁻¹ cm⁻¹ for 4A' and 4B, respectively (Patel et al., 1992). In all cases gene 4 protein concentrations are reported as monomer concentration. Nuclease S7, calf intestine phosphatase, and terminal transferase enzymes were purchased from Boehringer Mannheim. All gel-filtration marker proteins were purchased from Sigma.

Oligodeoxynucleotides—Oligodeoxynucleotides were synthesized at the Biochemical Instrument Center at the Ohio State University. The sequence of 60-mer was 5'-ATTTC GTAAT CATGG TCATA CACAT AGATT TTCTT CGTAC ATAGT GTTCG TCAAG CGTGG TTCTT CAGTG ACACT GGTTT TCTCG ACTGT AGCAG GAGTC GTACT ATGTC AGCGT CAGTG TCGGT AGGTT GACGG TACGG CAGTG CTGCT TCGTG CAGTG. The 60-mer was purified on a 12% polyacrylamide, 7 M urea gel and the 30-mer on a 16% gel. The products were resolved on an 8% polyacrylamide, 7 M urea sequencing gel. Oligodeoxynucleotides were ethanol-precipitated with sodium acetate, and stored at −20°C.

Nuclease S7, calf intestine phosphatase, and terminal transferase enzymes were purchased from Boehringer Mannheim. All gel-filtration marker proteins were purchased from Sigma.

Data Analysis—All calibration plots were prepared by linear least squares analysis of the data. Unknown molecular masses of protein and unknown lengths of DNA were calculated from the slope and intercept values obtained from the linear fits of the respective calibration plots. Standard errors of the mean are reported as 95% confidence intervals.

**Cross-linking**—The bifunctional cross-linking reagent DMS was used for protein cross-linking studies. DMS was always prepared immediately before use by dissolving 30 mg of DMS in 100 μl of ice-cold TEA-HCl (0.15 M, pH 8.2). The pH of the DMS solution was readjusted to 8.2 with 1 M NaOH. Cross-linking was initiated by adding DMS (10 mg/ml) to 4A' or 4B protein (1-10 μM) that was preincubated in 50 mM TEA buffer, pH 8.2, that contained 10 mM MgCl2, 100 mM NaCl, and 1 mM DTT at 22°C. The cross-linking reactions were stopped after intervals ranging from 1 to 20 min by adding equal volume of 1 M glycine to aliquots of the reactions. The quenched samples were mixed with SDS sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 2% mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue), heated at 90°C for 2 min, and subjected to electrophoresis on a 3.8% SDS polyacrylamide gel with a 3% stacking gel using the buffer formulations of Laemmli (1970). Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

**Uniformly 32P-labeled poly(dT) DNA** was prepared by elongating a 30-mer (0.7 μM) in 22°C in the presence of MgTP-PCP (1 mM) and DNA nucleoside. A fixed concentration of poly(dT) (0.2 μM) was incubated with varying amounts of 4A' or 4B proteins (5-30 μM) at 22°C in the presence of MgTP-PCP (1 mM) and DNA nucleotides containing 20 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 2.5 mM CaCl2, 1 mM DTT, and 50 mM NaCl. Reactions were initiated by adding endonuclease S7 to a final concentration of 1.8 μg/ml and stopped 30 min later with SDS (1%). An equal volume of sequencing loading buffer (90% formamide, 1 mg/ml bromophenol blue, 1 mM EDTA) was added, and the samples were heated at 90°C for 2 min before electrophoresis. The products were resolved on an 8% polyacrylamide, 7 M urea sequencing gel. A 5'-radiolabeled 60-mer and 30-mer were added to each sample as size markers. DNA markers V (Boehringer Mannheim) were loaded on the same gel as size markers. DNA markers V were dephosphorylated with calf intestine phosphatase prior to 5'-radiolabeling with [γ-32P]ATP using T4 polynucleotide kinase. The products were visualized after electrophoresis by autoradiography.

**High Pressure Small-zone Gel-filtration**—Oligomerization of 4A' and 4B proteins in the presence of 1) MgTP-PCP, 2) MgTP-PCP plus DNA, and 3) in the absence of ligands was investigated by small-zone gel-filtration chromatography. Gel-filtrations were performed at 22°C using the standard elution buffer, 50 mM Tris-Cl, pH 7.5, 10 mM NaCl, and 0.1 mM EDTA. When studies were carried out to determine the effect of ligand binding, 10 mM MgCl2 and 100 mM dTMP-PCP were added to the standard elution buffer. Proteins at increasing concentrations (0.2-216 μM) were equilibrated in the elution buffer for at least 15 min before injection. Flow rates of 0.5 ml/min were used, and a 15-μl aliquot of each sample was routinely injected from a 50-μl injection loop. The oligomers were resolved on a 30-cm HPLC gel-filtration column (Bio-Sil SEC 400, Bio-Rad) that was pre-equilibrated in the elution buffer. Chromatography was performed on a Waters model 825 LC system, and proteins were detected using a Waters 470 scanning fluorescence detector (Millipore). The fluorescence detector provided the sensitivity needed to detect low amounts of protein, especially when nucleotides present in the elution buffer contributed significantly to the background absorbance. Proteins were selectively detected by monitoring the fluorescence of the protein tryptophan and tyrosine residues exciting the samples at 280 nm and monitoring emission at 340 nm. The following proteins were used as molecular mass standards: α-
crystallin (810 kDa), thyroglobulin (669 kDa), β-galactosidase (520 kDa), urease (480 kDa), catalase (232 kDa), and β-amylase (200 kDa). Blue dextran (2000 kDa) was used to determine the void volume and tyrosine (180 Da) to determine the included volume. The partition coefficient \( K_p \) of each protein was calculated as \( (V_e - V_i)/(V_e - V_i) \), where \( V_e \) is the elution volume of the protein, \( V_i \) is the void volume, and \( V_0 \) is the included volume. The apparent molecular masses (\( m_{app} \)) of 4A' and 4B protein oligomers were calculated from interpolation of a semilog plot of partition coefficients of the markers versus the molecular masses of the markers.

The only difference appeared to be in the cross-linking patterns of 4A' protein, 4B protein cross-linked in a similar manner and formed dimers and trimers after limited cross-linking and higher oligomers after longer period of cross-linking with DMS. However, unlike the 4A' protein, 4B forms relatively small amounts of oligomers beyond hexamers. After a longer period of cross-linking, hexamers are clearly the predominant species in 4B.

4A' and 4B proteins were cross-linked also in the presence of 1 mM Mg\(_{2+}\)TMP-PCP, 2 mM Mg\(_{2+}\)TMP-PCP plus 60-mer DNA, and 3) in the total absence of added ligands. Similar cross-linking patterns were obtained under the various conditions (data not shown). Oligomeric species ranging from dimers to hexamers were formed with similar kinetics as shown in Fig. 1. The only difference appeared to be in the cross-linking pattern of 4A' incubated in the presence of 1 mM Mg\(_{2+}\)TMP-PCP. Although hexamers were the major species in the presence of ligands and in the presence of magnesium, significant amounts of oligomers beyond hexamers were formed in 4A' in the presence of Mg\(_{2+}\)TMP-PCP. This was, however, not the case with 4B. Even in the presence of Mg\(_{2+}\)TMP-PCP, 4B cross-linked only up to the hexamer. Interestingly, when DNA was added to 4A', cross-linked species beyond hexamers reduced back to the levels seen with magnesium alone. Cross-linking was also performed in the presence of 1% SDS. Under these conditions, protein-protein interactions were completely disrupted, and no cross-linked species were observed even after 30 min of reaction with DMS (data not shown).

The cross-linking time courses of the gene 4 proteins show multiple bands of dimers and trimers which are better resolved in reactions cross-linked for 1 to 2 min (lanes 2 and 3 in Fig. 1, A and B). There are two to three types of dimers and at least three types of trimers clearly separated. The two dimer bands in 4B are of almost equal intensity, whereas the dimers in 4A' appear as bands of unequal intensities. This cross-
linking pattern contains important information related to the symmetry of the hexamer. However, the cross-linking data alone are insufficient to provide structural information, because similar multiple bands can originate from cross-linking of several hexamer structures. The two dimers, for instance, may have formed when two different dimer interfaces are cross-linked or represents two types of linkages on the same dimer interface. Similarly, the third dimer band may originate from a third type of dimer interface or may actually correspond to a doubly cross-linked species. Determination of the symmetry of the protomers in the hexamer must await more detailed analysis of the structure.

Small-zone Gel-filtration in the Absence of Ligands—The oligomeric structure of the gene 4 proteins was investigated in greater detail using high pressure small-zone gel-filtration chromatography. Gene 4 proteins were examined by gel-filtration over a wide range of protein concentrations, both in the absence and in the presence of ligands such as nucleotides and DNA. Gel-filtration studies in the absence of ligands were performed in the standard elution buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 0.1 mM EDTA). Experiments were carried out at 22 °C, and the apparent molecular masses (mapp) were calculated from interpolation of a semilog plot of partition coefficient (Kp) of the protein markers versus molecular masses. The elution profiles of 4A' in the absence of ligands at protein concentrations ranging from 0.7 to 216 μM are shown in Fig. 2A. 4A' elutes as two peaks between concentrations of 0.7 μM to around 40 μM; the mapp of the faster eluting peak at 0.7 μM protein was calculated to be 572 ± 17 kDa, and the mass of the slower peak is 176 ± 10 kDa. The mapp values suggest that the first peak corresponds to an oligomer that contains nine 4A' monomers, whereas the second peak would contain 2.6 monomers. As the 4A' concentration is increased, the higher mass peak (faster migration) remains essentially unchanged and no new peaks appear. Yet the peak corresponding to the smaller species (slower migration) becomes broader and steadily moves toward the first peak. By 100 μM, the slower peak merges with the higher mass peak. This result is shown in the form of a plot in Fig. 2B, where the mapp values of the migrating peak are plotted against log of 4A' concentrations. The fact that we see a single peak throughout the concentration range (except for the higher mass peak that remains unchanged) indicates that at any given protein concentration 4A' contains a mixture of oligomers the subunits of which are in equilibrium with each other and exchanging on a time scale faster than the elution time. The position of the peak would thus be a function of the weighted average of the masses of the various oligomeric species. Even at 216 μM, the highest 4A' protein concentration used in these experiments, the protein peak is asymmetric, suggesting that 4A' may still continue to aggregate into higher oligomers as its concentration is increased.

4B protein assembled in a similar manner as its concentration was increased (data not shown), although no high mass species similar to the one observed in 4A' was present at lower 4B concentrations. 4B appeared to form only dimers at lower concentrations, and at higher concentrations, the dimer peak became broader and steadily eluted faster as observed with 4A'.

Small-zone Gel-filtration in the Presence of Mg2TMP-PCP—The effect of nucleotide binding on oligomerization of the gene 4 proteins was studied by using dTMP-PCP, the nonhydrolyzable analog of dTTP. The analog was used in all our experiments to avoid complications resulting from hydrolysis of dTTP. It was not possible to study the effect of Mg2+ in the absence of ligands using gel-filtration, because the gene 4 proteins precipitate in the presence of Mg2+ and remain irreversibly bound to the gel-filtration column. Gel-filtration experiments in the presence of Mg2TMP-PCP were carried out by adding the desired concentration of dTMP-PCP to the standard elution buffer that contained 10 mM MgCl2. Various dTMP-PCP concentrations ranging from 25 to 200 μM were used. The elution profiles of 4A' were nearly identical above 25 μM, hence detailed studies were conducted at 100 μM dTMP-PCP. Fig. 3A shows the elution profiles of 4A' at increasing protein concentrations (0.2–50 μM) in the presence of Mg2TMP-PCP. 4A' at 0.2 μM elutes as two distinct peaks. The mapp of the faster peak was experimentally determined to be 414 ± 16 kDa and the mapp of the slower peak, 165 ± 18 kDa. The mapp values suggest that the faster peak contains 6.6 monomers, hence it could be a hexamer or a heptamer; and the slower peak contains 2.6 monomers, therefore it is either a dimer or a trimer.
Hexameric Structure of T7 Gene 4 Proteins

To establish the identity of the two peaks, we used DMS-cross-linked 4A' protein as a size marker. 4A' was cross-linked for 30 min in the presence of Mg\(^{2+}\) as described under "Experimental Procedures." Fig. 4A shows distribution of the various cross-linked species resolved by SDS-polyacrylamide gel electrophoresis; the predominant species is the 4A' hexamer with a significant amount of dimer also present. Both uncross-linked and cross-linked 4A' proteins were applied to the gel-filtration column, and chromatography was carried out using the standard elution buffer in the absence of MgdTMP-PCP. Fig. 4B compares the elution profile of 4A' in the presence of MgdTMP-PCP with that of cross-linked and uncross-linked 4A' protein. Untreated 4A' at 10 \(\mu\)M protein concentration elutes as two peaks (Fig. 4B, profile 3). The slower peak which is the major species has an \(m_{\text{app}}\) of 229 \(\pm 13\) kDa (trimer or a tetramer) and the faster peak which is the minor species has an \(m_{\text{app}}\) of 575 \(\pm 23\) kDa. The elution profile of cross-linked 4A' (Fig. 4B, profile 2) is different from the uncross-linked protein. The cross-linked protein shows a number of species. Species that elute in the void volume are most likely highly cross-linked aggregates, and the two peaks that follow have \(m_{\text{app}}\) values equal to 472 \(\pm 20\) kDa and 176 \(\pm 15\) kDa. The 472-kDa peak migrates only slightly faster than the major species in the presence of MgdTMP-PCP (Fig. 4B, profile 1). Similarly, the 176-kDa peak migrates closer in time scale to the minor slower peak in the presence of MgdTMP-PCP. Based on these results, we assign the faster eluting species of 4A' in the presence of MgdTMP-PCP as the hexamer and the slower peak as the dimer.

4A' protein oligomerization in the presence of MgdTMP-PCP was investigated at protein concentrations ranging from 0.2 to 50 \(\mu\)M in the presence of single-stranded DNA (Fig. 5).

Fig. 3. High pressure gel-filtration analysis of 4A' and 4B proteins in the presence of MgdTMP-PCP. The effect of nucleotide binding on oligomerization of the gene 4 proteins was investigated by gel-filtration using the standard elution buffer (50 mM Tris-Cl, 50 mM NaCl, and 0.1 mM EDTA) to which 10 mM MgCl\(_2\) and 100 \(\mu\)M dTMP-PCP were added. 4A' and 4B proteins were preincubated in the elution buffer and gel-filtration was performed at 22 °C. Proteins were selectively detected by continuous monitoring of intrinsic protein fluorescence. A, elution profiles of 4A' at 0.2, 1, 4, 8, and 50 \(\mu\)M protein concentrations. The \(m_{\text{app}}\) values were calculated from interpolation of the standard calibration plot. The \(m_{\text{app}}\) of the faster eluting 4A' species (16.4 min) at 0.2 \(\mu\)M protein is 414 \(\pm 16\) kDa, and the \(m_{\text{app}}\) of the slower peak (19.2 min) is 165 \(\pm 18\) kDa. As 4A' concentration is increased the faster peak elutes at the same position, and the slower peak gradually decreases in intensity. A small peak eluting faster than the major species has an \(m_{\text{app}}\) of 752 \(\pm 14\) kDa. B, elution profiles of 4B at 0.5, 5, and 50 \(\mu\)M protein in the presence of MgdTMP-PCP. The \(m_{\text{app}}\) values of the two peaks at 0.5 \(\mu\)M protein concentration eluting around 17 and 19.6 min are 340 \(\pm 10\) and 144 \(\pm 12\) kDa, respectively.

The values of the protein peaks were highly reproducible, the uncertainty in the assignment of the oligomeric species arises from the fact that the \(m_{\text{app}}\) values were calculated by assuming that the gene 4 proteins are globular or similar in shape to the standard proteins. Therefore, a systematic error can be introduced if the shape of the gene 4 proteins is different from the standard proteins.

Fig. 4. Comparison of the high pressure gel-filtration profile of DMS-cross-linked 4A' to uncross-linked protein and 4A' in the presence of MgdTMP-PCP. The elution profiles of cross-linked and uncross-linked 4A' are compared with that of 4A' in the presence of MgdTMP-PCP. 4A' (10 \(\mu\)M) was cross-linked with DMS for 30 min as described under "Experimental Procedures." Both cross-linked and uncross-linked 4A' protein oligomers were resolved by gel-filtration using the standard elution buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 0.1 mM EDTA). A, the 3.8% polyacrylamide gel shows size markers (lane 1) and cross-linked species of 4A' (lane 2). B, gel-filtration profile 1 shows 4A' species at 10 \(\mu\)M concentration in the presence of MgdTMP-PCP (100 \(\mu\)M). The \(m_{\text{app}}\) of the major peak eluting at 16.4 min is 414 \(\pm 16\) kDa, and the two minor peaks eluting at 14.6 and 19.2 min have \(m_{\text{app}}\) values equal to 752 \(\pm 14\) and 165 \(\pm 18\) kDa, respectively. Profile 2 shows DMS-cross-linked 4A' protein at 10 \(\mu\)M resolved by gel-filtration in the absence of MgdTMP-PCP. The \(m_{\text{app}}\) values of the two major peaks eluting at 16 and 18.8 min are 472 \(\pm 20\) and 176 \(\pm 15\) kDa, respectively. Profile 3 shows 10 \(\mu\)M of untreated 4A' resolved by gel-filtration in the absence of MgdTMP-PCP. The \(m_{\text{app}}\) values of the two uncross-linked 4A' peaks eluting at 15.8 and 18.2 min are 575 \(\pm 23\) and 229 \(\pm 13\) kDa, respectively.
almost equal amounts of dimers and hexamers are present at the slower peak eluting at 20.3 min.

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FIG. 5. High pressure gel-filtration analysis of 4A' and 4B proteins in the presence of MgdTMP-PCP and single-stranded DNA. The effect of single-stranded DNA binding on oligomerization of the gene 4 proteins was studied by gel-filtration using the standard elution buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA) that contained 10 mM MgCl₂ and 100 μM dTMP-PCP. Proteins at various concentrations were incubated in the elution buffer in the presence of a constant amount of 60-mer (40 μM). Proteins were selectively detected by continuous monitoring of intrinsic protein fluorescence. A, elution profiles of 4A' protein at 0.1, 1, 4, 8, and 50 μM concentrations. The mₚₑₚ of the faster eluting peak (16.4 min) at 0.2 μM 4A' concentration was calculated as 420 ± 20 kDa, whereas the slower peak eluting at 20.3 min (*) corresponds to free 60-mer. B, elution profiles of 4B at 0.5, 5, and 50 μM protein concentrations. The mₚₑₚ of the faster eluting peak (17 min) at 4B concentration of 0.5 μM is 340 ± 10 kDa, and the slower peak eluting around 20.3 min (*) corresponds to free DNA.

and in the absence of DNA (Fig. 3). As shown in Fig. 3A, almost equal amounts of dimers and hexamers are present at 0.2 μM of 4A' in the presence of MgdTMP-PCP. As 4A' concentration is increased, more dimers are converted into hexamers, and around 8 μM, only 4A' hexamers are present. As protein concentration is increased even further, the hexamer peak becomes more symmetrical and a small peak with mₚₑₚ of 752 ± 15 kDa begins to appear. The mₚₑₚ of this new peak is very close to a 4A' dodecamer. Gel-filtration analysis of 4B in the presence of 100 μM dTMP-PCP was carried out at a few different concentrations as shown in Fig. 3B. Similar concentration dependence was observed in 4B. At 0.5 μM, some dimers (144 ± 12 kDa), but mostly hexamers (340 ± 10 kDa), are present. At 4B concentrations of 5 and 50 μM, hexamers are the predominant species. A small amount of 4B dodecamer (614 ± 11 kDa) also begins to appear at 50 μM 4B concentration.

Nucleotide Protection of Single-stranded DNA—Hexameric helicases such as E. coli DnaB (Arai and Kornberg, 1981) and Rho protein (Bear et al., 1988) that are closer in size to the gene 4 proteins have been reported to bind and protect between 60 and 80 bases of single-stranded DNA and RNA, respectively. We have also used nucleic acid protection experiments to determine the length of single-stranded DNA that is protected by the gene 4 protein oligomers. The DNA substrate used in these experiments was a uniformly radiolabeled single-stranded poly(dT). The radiolabeled poly(dT) DNA (≥500 bases long) was preincubated with 4A' or 4B protein in the presence of MgdTMP-PCP. The unprotected single-stranded DNA regions were digested with endonuclease S7. Fig. 6, A and B, show, respectively, the protection products of 4A' and 4B proteins resolved on sequencing gels. Instead of

M. M. Hingorani and S. S. Patel, unpublished results.
Hexameric Structure of T7 Gene 4 Proteins

Both protein cross-linking and gel-filtration results presented here provide evidence for hexamerization of the gene 4 proteins. Protein cross-linking of the gene 4 proteins with DMS resulted in oligomers ranging from dimers to hexamers and beyond. The kinetics of cross-linking showed that the predominant species to accumulate after extensive cross-linking were the hexamers of 4A' and 4B proteins. A more detailed study of the self-assembly process was carried out using small-zone high-pressure gel-filtration chromatography. 4A' and 4B proteins were examined at increasing protein concentrations, both in the absence and in the presence of MgTMP-PCP and DNA. In the absence of ligands, gene 4 proteins appear to form dimers or trimers at lower protein concentrations, and as protein concentration was increased, higher oligomers are formed. Protein concentrations as high as 100 μM were required to form stable higher oligomers in the absence of ligands. The gel-filtration behavior of the gene 4 proteins also indicates that the oligomers are relatively unstable and the subunits exchange on a time scale probably faster than the gel-filtration elution time of about 20 min.

In the presence of MgTMP-PCP, both 4A' and 4B proteins form species that appear to be hexamers at a much lower protein concentration. Therefore, although close to 100 μM protein concentrations were required to form higher oligomers in the absence of ligands, addition of MgTMP-PCP allowed oligomer formation at concentrations less than 8 μM. Similar effects of MgNTP binding on oligomerization have been observed in helicases such as E. coli DnaB (Arai et al., 1981), Polyoma large T antigen (Wang and Pnves, 1991), and E. coli rho protein (Finger and Richardson, 1982). MgATP binding in these helicases was shown to facilitate hexamer formation. Exactly how MgNTP induces hexamer formation in these helicases is not clear. MgNTP binding could induce conformational changes in the helicase that may facilitate assembly or NTP binding may actually stabilize the hexamer structure. Because a nonhydrolyzable analog is able to induce oligomer formation, NTP hydrolysis apparently is not required for hexamer formation. MgNTP-induced conformational changes have been observed by changes in the proteolysis pattern of helicases such as E. coli DnaB (Nakayama et al., 1984) and E. coli Rep (Chao and Lohman, 1990).

The presence of single-stranded DNA in addition to MgTMP-PCP induced hexamer formation at a much lower protein concentration. Thus, in the presence of a 60-mer single-stranded DNA hexamers were the only species at 0.2 μM protein concentrations were required to form higher oligomers in the absence of ligands, addition of MgTMP-PCP allowed oligomer formation at concentrations less than 8 μM. Similar effects of MgNTP binding on oligomerization have been observed in helicases such as E. coli DnaB (Arai et al., 1981), Polyoma large T antigen (Wang and Pnves, 1991), and E. coli rho protein (Finger and Richardson, 1982). MgATP binding in these helicases was shown to facilitate hexamer formation. Exactly how MgNTP induces hexamer formation in these helicases is not clear. MgNTP binding could induce conformational changes in the helicase that may facilitate assembly or NTP binding may actually stabilize the hexamer structure. Because a nonhydrolyzable analog is able to induce oligomer formation, NTP hydrolysis apparently is not required for hexamer formation. MgNTP-induced conformational changes have been observed by changes in the proteolysis pattern of helicases such as E. coli DnaB (Nakayama et al., 1984) and E. coli Rep (Chao and Lohman, 1990).

The presence of single-stranded DNA in addition to MgTMP-PCP induced hexamer formation at a much lower protein concentration. Thus, in the presence of a 60-mer single-stranded DNA hexamers were the only species at 0.2 μM, the lowest 4A' protein concentration that we could examine. We have not made any attempts to measure the association constants for hexamer formation because of the uncertainty in the measurement using small-zone gel-filtration. Accurate estimation of the association constants will require both the use of lower protein concentrations and a method such as broad-zone gel-filtration (Valdes and Ackers, 1979). Judging from the concentration dependence of oligomer formation, the association constant for oligomer formation in the presence of DNA is probably greater than 5 × 10^10 M^-1. DNA binding also appears to stabilize the hexamer structure and prevents free exchange of subunits between oligomers. This could explain our earlier observation where the primase activity of 4A' protein that was preincubated with dTTP and single-stranded DNA could not be activated by 4B (Patel et
Activation was observed only when 4B was mixed with 4A' prior to addition of DNA. If the mechanism of primase activation required 4B to form mixed oligomers with 4A', then preincubation with DNA would prevent free exchange of 4A' subunits with 4B, and the addition of 4B would have no effect on the primase activity of 4A'.

Both 4A' and 4B proteins were found to have similar oligomerization properties. The only difference appears to be the greater tendency of 4A' to form oligomers beyond hexamers as compared with the 4B protein. It is, therefore, very likely that the N-terminal 63 amino acids absent in 4B are not involved in direct protein-protein interaction. Gene 4 proteins appear to share many properties with helicases that have been reported to form hexamers, including E. coli Rho and DnaB proteins, as well as the large T antigen. Hexamer formation is induced in these helicases by MgNTP binding. Similarly, DNA binding appears to stabilize the hexamer structure of helicases such as Rho and T antigen. All of these helicases, including gene 4 proteins, also bind single-stranded DNA and protect between 60 and 80 bases. Recently, gene 4 proteins were shown to share regions of amino acid homology with bacterial DnaB helicases and bacteriophage T4, T3, and P22 helicase proteins (Ilyina et al., 1992). Most of these helicases are involved in DNA replication, and the bacterial DnaB helicases and T7 DNA helicase are known to form hexamers. Whether other helicases in this family also share a similar structure remains to be determined. Similarly, it is not known whether helicases that assemble into hexamers employ a common unwinding mechanism that is different from the mechanisms employed by dimeric helicases and helicases thought to be monomeric.

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REFERENCES


