Interaction of a DNA-binding Protein, the Gene Product of D5 of Bacteriophage T5, with Double-stranded DNA

ANALYSIS BY METRIZAMIDE GRADIENT CENTRIFUGATION*

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Interactions of DNA and the gene product D5 (gpD5) of bacteriophage T5, a DNA-binding protein that binds preferentially and cooperatively to double-stranded DNA, were analyzed by metrizamide gradient centrifugation. Conditions were set so that DNA and DNA protein complex sedimented to apparent equilibrium positions. DNA has a buoyant density of 1.12 g/cm³, and DNA saturated with gpD5 has a buoyant density of 1.17 g/cm³. These values are independent of DNA size and base composition in the range studied. At gpD5 concentrations below the saturation value in a low ionic strength buffer, DNA distribution is bimodal, indicating cooperative binding of gpD5 to DNA. However, in the presence of 10 mM MgCl₂, the binding process becomes distributive, with the buoyant density increasing linearly with the amount of gpD5 added until the saturation. From these data, one molecule of gpD5 is calculated to cover 40 base pairs at saturation. The technique as described has general applicability to the study of any interaction between DNA and DNA-binding proteins that bind in sufficient amount to cause detectable changes in buoyant density.

In bacteriophage T5, only two genes are known to code for products absolutely essential for DNA replication (1); these are DNA polymerase, the product of genetic loci D7-D9 (2) and a DNA-binding protein that is the product of genetic locus D5 (3, 4). There are mutants of other genetic loci that affect the onset and duration of DNA synthesis and the rates of synthesis and degradation of DNA (1).

T5 DNA polymerase has several unique properties (5): it is the most processive enzyme among the polymerases purified to apparent homogeneity; it binds preferentially to a primer end of poly(dA-dT)·oligo(dT); and it is capable of synthesis from a "nick" as it displaces the strand ahead of a growing fork.

Gene product D5 has a dual role in DNA replication and control of transcription (6). It is essential for shutoff of some early transcriptions and for the expression of late genes (3, 4, 6), and it has been purified to apparent homogeneity and characterized (4). It is an asymmetric protein of M₀ = 28,900. There are about 500,000 copies/cell, making it the most abundant DNA-binding protein synthesized in T5 phage-infected cells. It binds to both double- and single-stranded DNA, but with higher affinity and cooperatively to double-stranded DNA. Thus, it differs from bacteriophage T4 gp32 and Escherichia coli single-strand-binding protein, which are known as helix-destabilizing proteins; these bind preferentially and cooperatively to single-stranded DNA (for a recent summary on these proteins, see Ref. 7).

Our preliminary work suggested that gpD5 has an essential role in the control of complex formation between DNA and DNA polymerases, but not in too obvious a manner. These observations induced us to investigate further the interaction of gpD5 with native DNA.

As shown in this communication, velocity sedimentation to apparent equilibrium in a metrizamide gradient is a simple method for studying aspects of DNA-protein interactions. With this technique, conditions for cooperative binding of gpD5 to duplex DNA are studied. DNA and DNA saturated with gpD5 sediment to apparent isopycnic points of 1.12 and 1.17 g/cm³, respectively. The saturated complex is calculated to have 1 protein/40 bp independent of DNA size and base composition.

MATERIALS AND METHODS³

Strains of Bacteria and Bacteriophages—TSHA 23, a mutant that lacks genetically determined single strand breaks, was a gift of Marc Rhoades, Johns Hopkins University (8). Sources of all the other bacteria and phages were given previously (9).

DNA Preparations—Both labeled and unlabeled T5 DNA were extracted from corresponding purified phages as described earlier (10). The intactness of T5 DNA was tested by alkali sucrose gradient centrifugation (9). PM2 [²H]DNA was prepared as described in Ref. 9. Restriction enzyme digests of DNAs were prepared essentially according to commercial sources, but the optimum amount was determined for each lot of enzymes by agarose gel electrophoresis of the products. Smal came from Boehringer Mannheim, HindIII from Miles, HpaI from Miles or Bethesda Research Laboratories, and HpaII from Bio-Lab. Calf thymus DNA (p-L, Bio-chemicals) was made to 0.5 mg/ml in 75 mM Tris-HCl (pH 7.6), 20 mM NaCl and then denatured by boiling for 5 min. Poly(dA·dT) was prepared in S. Mitra's laboratory (ORNL) and kindly given to us.

Complex Formation between gpD5 and DNA—The complex was formed in the reac tion buffer, which is a modification of the system of Rigs et al. (18). The reaction was generally carried out in 200 μl containing the following: 67 mM Tris-HCl (pH 7.6), 10 mM dithiothreitol.

³ Portions of this paper (including part of "Materials and Methods" and Fig. 1) are presented in miniprint at the end of the paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the American Chemical Society, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 2-0069, cite authors, and include a check or money order for $9.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: gpD5, product of gene D5; bp, base pairs; NaDodSO₄, sodium dodecyl sulfate; Temed, N,N,N',N'-tetramethylenediamine.

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Fig. 2. Formation of complexes gpD5 and T5 DNA and between gpD5 and T5 DNA restriction enzyme digests. Complexes between 3.6 μg of [32P]DNA (specific activity, 1700 cpm/μg) and varying amounts of gpD5 were analyzed by metrizamide gradient centrifugation as described under "Materials and Methods." T5HA DNA (column 1) and its restriction enzyme digests (HindIII (column 2) and HpaI (column 3)) were mixed with an increasing amount of gpD5 (top row, 0 μg; middle, 1.8 μg; bottom, 5.4 μg). Buoyant densities at the peaks are indicated by arrows in column 3. They are the same in other columns. One hundred μl of each fraction were counted. The average sum of radioactivity (cpm/100 μl) for each gradient was 2930 ± 130.

Metrizamide Gradient Centrifugation—The linear gradient was made with 16 and 40% (w/v) metrizamide (analytical grade, Nyegaard & Co.) in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.0), 5 mM ethylene glycol bist(β-aminoethyl ether)-N,N,N',N"-tetraacetic acid (both from Sigma). Centrifugation was for 16 h at 4 °C in an SW 50.1 rotor at 45,000 rpm or in an SW 65 rotor at 50,000 rpm. Results were indistinguishable. The buoyant densities of the metrizamide gradients were determined from the refractive index by the equation of Birnie et al. (19). Results were plotted by a computer using the program developed by Maddox Randal of ORNL.

Assay for DNA in Metrizamide Fractions—Radioactive DNAs, when free of acid-soluble fractions, were counted by use of INSTAGEL (Packard). Nonradioactive DNA concentrations were determined from fluorescence intensities. An aliquot of each fraction was acid-precipitated to remove metrizamide, and DNA was acid-hydrolyzed and reacted with diaminobenzoc acid (Aldrich) essentially as described by Setaro and Morley (20). The intensity of the fluorescence emission was measured at 510 nm (excitation wavelength, 420 nm) by use of an Amino-Boerman spectrofluorometer. Calf thymus DNA was used as a standard.

Gel Electrophoresis—Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit (Aqueboque Shop, NY) in the Tris-acetate system of Hayward and Smith (21). Agarose (Sea Kem) was usually 0.8% (w/v), and the run was made at a constant current of 30 mA for about 16 h. The procedure for polyacrylamide gradient gel electrophoresis is described in the Miniprint.

RESULTS

Analysis of gpD5-DNA Interactions by Metrizamide Density Gradient Centrifugation—Metrizamide density gradient centrifugation was chosen as a method for analysis of DNA-gpD5 complex because metrizamide is an inert, nonionic substance that does not interfere with assays of several enzymes involved in DNA metabolism. In metrizamide solutions, in contrast to CsCl solutions, the buoyant density of DNA is less than that of protein (19). Thus, the nucleoprotein has a higher buoyant density as more proteins complex with it. In our experiments, samples were layered on gradients and centrifuged until DNA and nucleoproteins sedimented to apparent equilibrium, but proteins sedimented only slightly because of their lower molecular weight. This interpretation is consistent with our results shown here.

First, complex formation between T5 DNA and gpD5 was studied by keeping the amount of DNA constant and changing the concentration of gpD5. As indicated in Fig. 2, DNA has buoyant density (ρ) of 1.12 g/cm³, in agreement with Birnie et al. (19). Complexes were formed in conditions similar to those of the nitrocellulose filter binding system of Rittig et al. (18), except that no Mg²⁺ was added. The filter binding assay had suggested that the complexes were formed very quickly at 0 °C, but samples were left standing for at least 10 min prior to being layered on the gradient. When varying concentrations of gpD5 were added, DNA was distributed in a bimodal fashion, with the height of the denser peak increasing with increasing concentration until all the DNA had shifted to that position (Fig. 2, column 1). The lighter peak was slightly denser than the DNA peak, and below 0.3 μg of gpD5, most of the DNA was in this position. The denser peak had a buoyant density of 1.17 g/cm³ and remained at this position even after addition of more gpD5.

These observations indicate that gpD5 binds initially to DNA in a distributive mode. As more gpD5 is added, a critical point is reached (ρ = 1.13 g/cm³), after which the additional amount of gpD5 binds cooperatively until the DNA becomes saturated, forming a denser peak at ρ = 1.17 g/cm³. All the DNA is saturated by 5.4 μg of gpD5/3.6 μg of DNA.

To study the influence of DNA size, T5 DNA was digested to smaller sizes with restriction enzymes. HindIII enzyme cuts T5 DNA into about 16 pieces (22, 23) of average Mₐ = 5 × 10⁶, SmalI cuts it into four pieces (22–24) of average Mₐ = 20 × 10⁶, and HpaI cuts the DNA into 26 pieces (24) of average Mₐ = 3 × 10⁶, assuming the molecular weight of T5 DNA to be 77 × 10⁶ (2). Columns 2 and 3 of Fig. 2 show the results with HindIII and HpaI digests (the smallest DNA we have studied), respectively. SmalI digests behaved similarly; bimodal shifts in buoyant density occurred at about the same protein/base pair ratios, and the complete shifts occurred in all cases by 5.4 μg of gpD5/3.6 μg of DNA. Recoveries of DNA from all

* R. K. Fujimura, unpublished observation.
the gradients were better than 93%. Areas under the corresponding peaks were about the same. Therefore, there was no obvious loss of certain fractions of DNA. In all cases DNA and DNA-gpD5 complex sedimented to the same respective positions ($\rho \approx 1.2$ and $1.17$ g/cm$^3$, respectively) independent of DNA size in the range of molecular weight studied.

Poly(dA-dT) sedimented to the same position as natural DNA, $\rho \approx 1.12$ g/cm$^3$ (Fig. 3). Thus, the equilibrium position is independent of base composition, as observed by Birnie et al. (19). The complex of poly(dA-dT) with gpD5 sedimented to the same position ($\rho \approx 1.17$ g/cm$^3$) as other DNA-gpD5 complexes. (There was considerable tailing of the peak, which may be due to some fractions of poly(dA-dT) being too small.) Results with poly(dA-dT) indicate that the position of the complexes in the gradient and the saturation point for binding are also independent of base composition.

These observations with restriction enzyme digests and poly(dA-dT) support the interpretation that the sedimentations of DNA and DNA-gpD5 complex were to apparent equilibrium points.

The essential requirements for cooperative binding of gpD5 to DNA are low ionic strength and absence of Mg$^{2+}$. Other ingredients in the reaction mixture (dithiothreitol, dimethyl sulfoxide, bovine serum albumin) had no obvious effect on the bimodal distribution of DNA (data not shown). Nor were there any obvious effects of temperature in the range of 0 to 37 °C (data not shown). Under the conditions for maximum binding (3.6 µg of DNA and 5.4 µg of gpD5), increasing the salt concentration decreased the density shifts (Fig. 4), indicating that less protein was bound as ionic strength was increased. In the presence of Mg$^{2+}$, density shifts of the complexes were gradual and unimodal, indicating distributive binding (Fig. 5). This was so even if Mg$^{2+}$ was added after the complex formation (Fig. 5F). However, the buoyant density of the complex at the saturation point was still 1.17 g/cm$^3$.

The data in the presence of MgCl$_2$ were used to estimate the number of base pairs/bound protein at the saturation point. As shown in Fig. 6, until near the saturation point, the distance shifted in the gradient was linearly proportional to the protein added. Extrapolation of the linear portion to the limit indicates that at the saturation point 4.8 µg or 0.16 nmol of gpD5 are bound per 3.6 µg or 6 nmol (base pairs) of DNA. Thus, the maximum capacity of binding is 1 molecule/38 bp. Density gradient fractions of the samples that had gpD5 just below the saturation point (sample A) and past the saturation point (sample B) were analyzed for the position of proteins. In sample A, gpD5 was detectable only in the position of the complex (Fig. 7A). In sample B, gpD5 was detectable both in the complex and at the meniscus (Fig. 7B), indicating gpD5 was in excess. This is consistent with our interpretation of the data. Rice et al. (4) also determined the saturation point, using $^{32}$P-gpD5 and sucrose density gradient centrifugation, to be 1 molecule/36 bp of T5 DNA, in close agreement with our figure, and showed that gpD5 was almost quantitatively bound until after the saturation point. However, we disagree with
their speculation that the saturation point differs among DNAs of different base compositions.

Gene product D5 is asymmetric with an axial ratio of 7.6:1 or 20 Å × 154 Å (4). The distance between base pairs of DNA in solution is 3.3 Å (25). Therefore, gpD5 could cover 47 bp, and at maximal binding T5 DNA may be in complex with a chain of gpD5 end to end. Cooperative binding at low ionic strength also suggests that gpD5 have either direct or DNA-mediated affinity for each other.

Binding to circular DNA was also tested with PM2 DNA as an example. The preparation had both superhelical and relaxed circles along with some linear DNA (see the inset to Fig. 8A). The DNA was also made linear by HpaII digestion, which cut the DNA once. There were no obvious differences in results. The saturated DNA had a buoyant density of 1.17 g/cm³, as did T5 DNA. Results with varying amounts of gpD5, but below the saturation point, are shown in Fig. 8. Any differences should be detectable at these transition ranges. PM2 DNA, bound to various amounts of gpD5, was isolated from the metrizamide gradient, recovered by phenol extraction, and analyzed by agarose-gel electrophoresis. About the same proportion of DNA was recovered as supercoiled and relaxed circles as from the original stock (data not shown). Therefore, circularity or superhelicity of DNA has no obvious effect on cooperative binding. The figure also shows that the lighter peak gradually increases in density during transition. This was also observed with T5 DNA.

**DISCUSSION**

The data presented suggest that DNA is saturated with gpD5 when there is 1 molecule for about 40 bp, which gives the buoyant density of 1.17 g/cm³ in metrizamide. T5 DNA has about $13 \times 10^4$ bp, and thus can be complexed with a chain of about 3000 gpD5. This means there are enough gpD5/cell to complex about 200 molecules equivalent to T5 DNA.

We have analyzed DNA-protein complex isolated from phage T5-infected cells, and shown that, at the late phase of infection where there is an abundance of gpD5, the complex has buoyant density of 1.17 g/cm³. Most of the DNA polymerase activities co-sedimented with it. Such fraction was absent in extracts from D5 mutant-infected cells. Thus, it is possible for many T5 DNAs to be in complex with a chain of gpD5. The effects of gpD5-DNA complexes on DNA polymerase functions have been characterized, in which it is shown that the amount of gpD5 binding to double-stranded regions affects synthesis with denatured DNA. In the presence of sufficient gpD5 to saturate the double-stranded regions, DNA synthesis is inhibited.

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5 R. K. Fujimura and B. C. Roop, manuscript in preparation.
Metrizamide gradient centrifugation as described here has general applicability to the study of any interaction between DNA and a DNA-binding protein that involves a sufficient number of proteins/DNA molecule to cause detectable changes in buoyant density. The advantage of the method is that the apparent equilibrium positions for DNA and DNA-protein complexes are independent of size. The method may be used to fractionate and study proteins that bind in clusters to specific sequences in a DNA fragment.

The theory of the mechanism of the binding of helix-destabilizing protein to DNA and repressor-DNA interaction is being developed to a sophisticated level (for recent developments, see Refs. 26, 27). Our observation is consistent with the theory as initially proposed (28) that the first step of protein binding is nonspecific adsorption to any part of the DNA that reduces the number of dimensions to one, the subsequent step being a sliding along the single dimension lattice. Thus, applying this to our system, gpD5 first binds to any part of DNA and then subsequently moves along the chain until it encounters another gpD5. At a certain point, about 300 mol of bp/mol of gpD5, the affinity of the gpD5 for DNA and a DNA-binding protein that involves a sufficient size specifically for the phage T5 DNA system is that it is unnecessary to maintain T5 DNA in intact form. We have used DNA from T5HA, a mutant that does not have site-specific breaks (8). However, for some reason, it tends to break to about half its size on storage. Concentrated mutant phage preparations tend to lyse in phosphate buffer under conditions at which the wild type is stable for months. Thus, by using the metrizamide gradient technique, we are free from the necessity of maintaining the DNA in intact form.

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REFERENCES
Preparation of DNA-free extracts - 15% selected cells (1g) were prepared from 15 liters of overnight culture and treated with a French pressure cell essentially as described previously (9). The disrupted cells were made to 2.5% (w/v) NaCl and centrifuged at 35,000 g for 30 min. For removal of DNA, the supernatant was made to 50% (w/v) polyethylene glycol 8000 (Union Carbide) and 5 min later was centrifuged at 35,000 g for 30 min. The supernatant fraction was discarded, the pellets washed three times with 0.01 M NaCl, and the washings were combined with the supernatant from the first wash. This protein frac was then dialyzed overnight in 3 liters of 0.01 M NaCl, and the dialysate was assayed for polymerase activity with dispersed calf thymus DNA to ensure that the cells were disrupted (9). The bound amount of protein was obtained.

Native DNA cellulose chromatography - Native DNA cellulose was prepared essentially as described by Littman and Albers (described in ref. (11)), and chromatography was carried out as described by Alberts and Herbst (12) in a 50-cm bed column volume. After adsorption of a sample and washing with 0.1 M NaCl/sodium phosphate buffer (pH 7) in buffer B containing (100 mm tris(hydroxymethyl)aminomethane, 50 mm KCl, and 1 mm MgCl2), the column was washed through the solvent gradient described in table 1. Fractions of 10 ml were collected. The last fractions contained DNA polymerase activity. The DNA polymerase activity was also lost at this step. The supernatant fraction was heated to the column and washed with buffer B. The sample was eluted with a linear gradient of 0 to 1 M NaCl in buffer B (1% per ml). Fractions around the 0.5 M NaCl region were collected on polyacrylamide gradient gel (about 300 ml). Those showing the parent gp70 were pooled (see Fig. 1A), dialyzed against buffer B, and stored at -20°C. Total protein content was 125 mg.

Desalted DNA cellulose chromatography - A desalted DNA cellulose column (15 ml bed volume) was prepared as described by Alberts and Herbst (12). The sample was eluted in 0.1 M NaCl/sodium phosphate buffer (pH 7) in buffer B containing (100 mm tris(hydroxymethyl)aminomethane, 50 mm KCl, and 1 mm MgCl2). Fractions of 10 ml were collected. Those fractions that showed only one band when about 25 mg of the proteins were run on polyacrylamide gradient gel electrophoresis were pooled, dialyzed against 10 volumes of buffer B/0.1 M NaCl, and stored at -20°C. The final protein contained 5% of gp70 with a molecular weight of approximately 20,000 in the Table B polyacrylamide gel electrophoresis, in agreement with Bieri et al. (1). Total protein content in the pooled fractions was 3.5 mg (Fig. 1A).

Characterization of Preparations - Polyacrylamide gradient gel electrophoresis in sodium dodecyl sulfate (SDS). A Stock solution of 10% sodium dodecyl sulfate in water. 10% polyacrylamide gel (thickness 0.5% w/v) was made and stored at 4°C. Linear gradients of 7.5% and 15% acrylamide were made by dialyzing the stock in a final concentration of 10% SDS, 5% (w/v) polyacrylamide, 30% (v/v) water. 10% polyacrylamide gel, and 10% acrylamide, and 20% acrylamide, respectively, for 48 h. The gel was then poured and dried at 90°C for 2 h. The sample was loaded as described by Bieri et al. (11). The electrophoresis was carried out at 4°C, 200 V, until bromophenol blue reached the bottom of the gel. The bands were stained with Coomassie blue (13) or silver stain (modification of the method of Marko et al., 14). SDS was used (Bio-Rad Laboratories, personal communication). The proteins of the preparations were shown in Fig. 1. Phosphorylation was no present band other than that for gp70 detected when 25 mg of the protein was used.

**Figure 1.** Polyacrylamide gradient gel electrophoresis of a gp70 preparative in sodium dodecyl sulfate. Details of the method are in the text. (A) 100 ml of samples from each fraction from the DNA-cellulose column were analyzed. 100 ml of protein from the DNA-cellulose step were analyzed. Fractions from the DNA-cellulose step were pooled and used for polyacrylamide gel electrophoresis. (B) DNA polymerase activity in each fraction from the DNA-cellulose step was analyzed. 250 ml of protein from each fraction from the DNA-cellulose step was analyzed. Each fraction from the DNA-cellulose step was analyzed. (C) DNA polymerase activity in each fraction from the DNA-cellulose step was analyzed. 250 ml of protein from each fraction from the DNA-cellulose step was analyzed. Each fraction from the DNA-cellulose step was analyzed. (D) DNA polymerase activity in each fraction from the DNA-cellulose step was analyzed. 250 ml of protein from each fraction from the DNA-cellulose step was analyzed. Each fraction from the DNA-cellulose step was analyzed.