DNA "Melting" Proteins

II. EFFECTS OF BACTERIOPHAGE T4 GENE 32-PROTEIN BINDING ON THE CONFORMATION AND STABILITY OF NUCLEIC ACID STRUCTURES*

(Received for publication, March 11, 1976)

DAVID E. JENSEN,† RAYMOND C. KELLY,§ AND PETER H. VON HIPPEL

From the Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403

Bacteriophage T4-coded gene 32-protein is an essential component of the T4 replication and recombination systems. Alberts and co-workers (Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D., and Ferris, F. L. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 289-305) have shown that the major physiological activity of the protein involves preferential and cooperative binding to single-stranded DNA. In this paper, the physicochemical parameters characterizing this "melting" protein system are quantitatively determined.

Boundary sedimentation velocity experiments are used to measure the interaction of gene 32-protein with native DNA. The binding is shown to be non-cooperative and involves an overlapping site size (n_h) of ~10 nucleotide residues (or ~5 nucleotide pairs). In analogy with the ribonuclease results (Jensen, D. E., and von Hippel, P. H. (1976) J. Biol. Chem. 251, 7198-7214), the logarithm of the association constant \( K_a \) is found to be linearly related to \( \log [Na^+] \).

The binding of gene 32-protein to denatured (single-stranded) DNA involves appreciable distortion of the polynucleotide backbone from the unliganded conformation; binding totally unstacks the bases of both ribose- and deoxyribose-containing polynucleotides at 10°, and results in a hyperchromic change exceeding which can be induced by heating. This hyperchromism induced in poly(dA) on binding gene 32-protein under low salt (tight binding) conditions is used to determine a value of \( n_s \) (the single-stranded DNA site size) of ~6.7 nucleotide residues per protein. In addition, gene 32-protein binding to single-stranded polynucleotide induces an unusual circular dichroic spectrum characterized principally by a marked decrease in the magnitude of the positive CD band centered at ~265 nm. This spectral change is attributed to significant uncoupling of the transition moments of the vicinal bases of the single-stranded polynucleotide on gene 32-protein binding, in accord with the ultraviolet hyperchromism observed. Binding of gene 32-protein to double helical DNA has virtually no effect on the spectral properties of this conformation.

Gene 32-protein-perturbed melting transitions of double helical poly[d(A-T)] are analyzed by the techniques of McGhee (McGhee, J. M. (1976) Biopolymers, 15, 1345-1375), to independently establish best fit values of \( n_c \), \( K_a \) (the association constant to single-stranded polynucleotide), and \( \omega_c \) (the protein interaction cooperativity parameter on single-stranded polynucleotides). Values of \( n_c \approx 7.5 \) nucleotide residues/protein monomer, \( K_a = 10^9 M^{-1} \) (at 0.01 M Na⁺), and \( \omega_c = 10^3 \) are measured for this system. \( K_a \) is estimated to be ~2 orders of magnitude larger than \( K_a \) at various ionic strengths while \( \omega_c \) appears to be fairly independent of salt concentration.

Although gene 32-protein is a very effective equilibrium destabilizer of double helical poly[d(A-T)], this type of equilibrium is not attained in melting experiments with a number of different natural DNAs, indicating that the destabilization of native DNAs by gene 32-protein is kinetically blocked. The nature and possible physiological significance of this kinetic block to native DNA destabilization by gene 32 protein is considered.

*This research was supported in part by United States Public Health Service Research Grants GM-15792 and GM-15423, as well as by Predoctoral Traineeships (to D. E. J. and R. C. K.) from United States Public Health Service Training Grant GM-00444. This work was submitted (by D. E. J.) to the University of Oregon Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Present address, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331.

§ Present address, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106.
In the preceding paper (1) we considered the effects on DNA conformation and stability of the binding to DNA of the model "melting" protein, ribonuclease. In this paper we extend these considerations to a "real" (physiologically relevant) DNA-melting protein system, examining the effects on nucleic acid structure of the binding of bacteriophage T4-coded gene 32-protein and exploiting the structural consequences of this binding to obtain direct measurements of the thermodynamic parameters characterizing this nucleic acid-protein interaction system.

The protein coded by gene 32 of phage T4 appears to be essential for successful lytic infection of Escherichia coli by this phage; i.e., amber or temperature-sensitive mutants of this gene have been shown to be defective in replication (2-6) and recombination (2, 7) functions. The protein is produced in large quantities throughout the lytic growth cycle of T4 (3, 5, 8-12) and various genetic differences have suggested that it might facilitate replication and recombination by binding to and stabilizing intermediate single-stranded states of the genome crucial to these information transfer processes.

This notion acquired a molecular basis when Alberts and co-workers (3, 4) demonstrated in vitro that gene 32-protein binds more tightly to single-stranded than to double-stranded DNA, and that the affinity for single-stranded DNA increases with increasing protein concentration, i.e., binding is cooperative. Cooperative binding facilitates saturation of single-stranded sequences at moderate protein concentrations (13). A demonstration that gene 32-protein molecules overlap when bound in contiguous and that stabilizing protein-protein interactions may be responsible for the observed cooperative binding. The reported tendency of gene 32-protein to undergo concentration-dependent self-aggregation in the absence of DNA may be a related phenomenon (4, 15). The suggestion that binding is cooperative has been supported by studies of gene 32-protein complexes with single-stranded DNA and gene 32-protein, that the association constant for gene 32-protein binding cooperatively (contiguously) to single-stranded DNA is greater than 10^13 M^-1 cm^3. Other native DNAs were obtained from Workington and routinely deproteinized as described previously (1). The following 260 nm extinction coefficients (per mol of phosphate residues) were used (21): calf thymus DNA, 6.5 x 10^5 M^-1 cm^-1; Clostridium perfringens DNA, 6.4 x 10^5 M^-1 cm^-1; and Micrococcus lysodeikticus DNA, 6.0 x 10^5 M^-1 cm^-1.

The synthetic polynucleotides poly(dA-dT), poly(dA), poly(dT), and poly(dT) were obtained from P-L Biochemicals. The concentrations of these polymers were determined by ultraviolet absorbance, using the following extinction coefficients (in moles of phosphate residues; M^-1 cm^-1): poly(dA), 10 x 10^5 at 257 nm (22); poly(dA), 10.5 x 10^5 at 257 nm (23); poly(dT), 8.1 x 10^5 at 260 nm (24); and poly(dT-T) 6.6 x 10^5 at 262 nm (25).

**Methods**—Details of most of the experimental methods used in this study are discussed in Jensen and von Hippel (1) and only additional procedures are described here.

**Microtechnique for Determination of Binding Parameters**—The limited quantities of gene 32-protein available necessitated a "scaling down" of the boundary sedimentation velocity technique for the determination of binding parameters. This microtechnique is essentially the same as the standard method described by Jensen and von Hippel (1), except that the volume of solution required for the assay is reduced, decreasing the quantities of DNA and protein required by ~80%. Micro-nitrocellulose centrifuge tubes (0.8 ml capacity) were obtained from Beckman, together with adaptors which allow these tubes to be used in the SW 50.1 rotor. The length of the small tube is about 80% that of the standard nitrocellulose tube, therefore requiring adjustments of the sedimentation run time. Upon completion of the run, the gradients were fractionated by the careful removal of 100-μl aliquots from the top of the tube, and the fractions diluted and assayed as described elsewhere (1).

**RESULTS**

**A. Thermodynamic Parameters Characterizing Binding of Gene 32-Protein to Native DNA**

Alberts and Frey (4) observed that the binding affinity of gene 32-protein for single-stranded DNA decreases gradually with increasing sodium chloride concentration over the range 0.15 to 0.60 M, and suggested that electrostatic interactions play a role in the binding reaction. They proposed that the region of the protein in direct contact with DNA must include a 5 D. E. Jeneen and P. H. von Hippel, submitted to Anal. Biochem.
gene 32-protein might also bind appreciably to the higher substantially with decreasing salt concentrations, and since DNA phosphates, even though gene 32-protein has a net "patch" of positively charge residues which interact with the and discussions in Ref. 1, as well as Footnote 2) that the negative charge at neutral pH (14). We have found (see below consideration of the effect of gene 32-protein on polynucleotide helix-coil transitions, requires a knowledge of the magnitude of binding of gene 32-protein to native DNA, we have made some preliminary determinations of the relevant binding parameters as a function of ionic strength. Because limited quantities of gene 32-protein were available, the "micro" version of the sedimentation velocity binding technique was used in these studies (see "Experimental Procedure") and only three points on each binding curve were determined at each of three salt concentrations. The three sets of data were plotted as \( v/L_{\text{free}} \) versus \( v \) and a straight line was fitted to each set (this approach assumes the "Scatchard approximation" of discrete and independent DNA binding sites; see Ref. 13). The data show no indication of binding cooperativity, in confirmation of the original suggestion of Alberts and co-workers (3, 4) that binding to native DNA is non-cooperative. The lines cross the \( x \) axis at about 0.05, indicating an uncorrected site size of 20 nucleotides (10 base pairs). Treatment of data for ligands showing "overlap" binding by this independent site method overestimates ligand binding site size by about a factor of 2 (13); thus, the native DNA site size is approximately 10 nucleotides (5 nucleotide pairs). This site size presumably reflects the packing of the protein on the outside of the DNA helix; although no geometric details are available, ionic strength effects on the binding constants support the notion that the primary interaction may involve binding of positive residues in the gene 32-protein-nucleic acid binding site to phosphate groups on the outside of the DNA double helix. Apparent binding constants for gene 32-protein to native DNA at various ionic strengths, measured as \( v/L_{\text{free}} \) intercepts on Scatchard plots, are summarized in Table 1. These values all represent slight underestimates (by 10 to 20%) of the actual binding constants, again due to neglect of the correction of "overlap" of potential binding sites (13), but should provide reliable estimates of the ionic strength (sodium ion concentration) dependence of the binding constant. Analysis of these data shows an approximately linear relationship between the logarithm of the binding constant and the logarithm of the sodium ion concentration, suggesting that the free energy of interaction of native DNA with gene 32-protein does indeed contain a significant electrostatic component. Furthermore, it is noted that the magnitudes of these binding constants are comparable to those measured for the interaction of ribonuclease with native and denatured DNA at comparable ionic strengths and that the ribonuclease-DNA systems also show linear (although somewhat steeper) log \( K \) versus log [Na+] plots (1). Record et al. have used these data to estimate the number of ion pairs involved in gene 32-protein binding to native DNA on the basis of a general thermodynamic treatment of charged ligand interactions with linear poly electrolytes. Their estimate of one to two DNA phosphates involved in the interaction is in good accord with the results of our measurements on the binding of short oligonucleotides to gene 32-protein (see Ref. 18).

### Table 1

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<thead>
<tr>
<th>[Na+] M</th>
<th>( K_{\text{M}} ) M⁻¹</th>
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<tr>
<td>0.020</td>
<td>3.9 ( \times ) 10⁴</td>
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<tr>
<td>0.030</td>
<td>1.9 ( \times ) 10⁴</td>
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<td>0.050</td>
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*Estimated from binding plots (\( v/L_{\text{free}} \) versus \( v \)) using the "Scatchard approximation," see text.

"patch" of positively charge residues which interact with the DNA phosphates, even though gene 32-protein has a net negative charge at neutral pH (14). We have found (see below and discussions in Ref. 1, as well as Footnote 2) that the electrostatic component of the free energy of binding increases substantially with decreasing salt concentrations, and since many of our measurements are carried out at sodium ion concentrations in the range 0.01 to 0.1 M, we anticipated that gene 32-protein might also bind appreciably to the higher charge-density native form of DNA over this range of salt concentrations. Since a complete description of the distribution of gene 32-protein in the cell, as well as quantitative consideration of the effect of gene 32-protein on polynucleotide helix-coil transitions, requires a knowledge of the magnitude of binding of gene 32-protein to native DNA, we have made some preliminary determinations of the relevant binding parameters as a function of ionic strength.

### Conformation of Single-stranded DNA Complexed with Gene 32-Protein

Alberts and Frey (4) showed that a saturated complex of gene 32-protein and single-stranded fd DNA sediments only ~1.3-fold faster than the protein-free DNA alone. Since the mass of the complex is 13- to 25-fold greater than that of the free DNA (depending upon the binding site size used, see below) and the buoyant density of the complex is only 30 to 40% less, this result showed that gene 32-protein binding must increase the frictional coefficient of single-stranded DNA several fold above the value characteristic of the uncomplexed strand. On this basis, Alberts and Frey concluded that DNA complexed with gene 32-protein must be held in a highly extended and rigid configuration. This inference is in accord with the electron microscopic studies of Delius et al. (16), who showed that gene 32-protein-coated single-stranded fd DNA appears structurally rigid and elongated, extending ~4.6 Å per nucleotide residue along the backbone direction. These results indicated that the conformation of single-stranded DNA complexed with gene 32-protein might be rather unusual and led us to investigate this conformation using ultraviolet absorbance spectrometry and circular dichroism.

### Ultraviolet Hyperchromicity of Polynucleotides Complexed with Gene 32-Protein

In the course of these studies, we observed that single-stranded polynucleotides complexed with gene 32-protein become appreciably hyperchromic. In fact, at saturating protein concentrations and room tempera

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...ture, this hyperchromism appeared to exceed that usually observed for the protein-free polymer at high temperature, suggesting that gene 32-protein binding fully unstacks the nucleotide bases and leaves even less residual base-base interaction than is present when the polynucleotide secondary structure is thermally disrupted. This effect has been investigated with several polynucleotides.

Fig. 1 shows the effects of binding increasing quantities of gene 32-protein on the ultraviolet absorption spectrum of single-stranded poly(dA) at 0.10 mM Na+ and 24°C; at this temperature and pH (7.7), uncomplexed poly(dA) exists primarily as single-stranded, non-cooperatively melting, stacked structures (26). Clearly, gene 32-protein binding increases the hyperchromicity of poly(dA) (Fig. 1A); we note that this increase is much greater than observed in poly(dA)·RNase complexes (1). Fig. 1B shows the difference spectra for these systems; i.e., the spectra of the poly(dA)·gene 32-protein complex minus those of the separate components under the same conditions. Qualitatively, the effect resembles the thermally induced unstacking of DNA, with little or no change in peak position and a relatively constant fractional increase in optical density at all wavelengths (27). Similar measurements were made of the effects of gene 32-protein binding on poly(rA), which also exists as a single-stranded, partially stacked structure under these conditions. The resulting spectra and difference spectra (data not shown) are virtually identical to those of Fig. 1, A and B.

Heat-denatured and reannealed calf thymus DNA also shows an appreciable hyperchromicity at room temperature relative to its ultraviolet absorbance spectrum at high temperature. This hyperchromicity also reflects base-stacking, but, in natural DNAs it has been shown that virtually all the stacked bases occur in double-stranded, interbase hydrogen-bonded structures (27, 28), most of which are probably short hydrogen-bonded “hairpin” loops of varying length and thermal stability. As is shown below, gene 32-protein readily denatures such structures; on binding, the denatured DNA spectrum becomes appreciably more hyperchromic (data not shown), indicating that complex formation with gene 32-protein disrupts the base-stacking of these hairpin loops as effectively as it does the non-cooperative single-stranded stacked structures of poly(dA) and poly(rA).

The synthetic co-polymer poly[d(A-T)] forms a stable double helical structure closely resembling native DNA at room temperature in the presence of 0.01 mM Na+; however, as shown in a subsequent section, it is fully (cooperatively) melted out in the presence of saturating amounts of gene 32-protein under these conditions. Fig. 2, A and B show that increasing amounts of gene 32-protein also render this polynucleotide hyperchromic on complex formation with the single-stranded form; this effect was exploited by Alberts and Frey (4) in studying Mg2+-induced renaturation of poly[d(A-T)] in the presence of gene 32-protein.

Further insight into these phenomena is obtained by examining the melting behavior of poly(dA), poly(rA), and denatured calf thymus DNA in the presence of gene 32-protein. All three systems show broad, non-cooperative melting profiles in the absence of protein (Fig. 3, solid lines); at room temperature poly(dA) and poly(rA) are about 50% stacked (27), while in 0.01 mM NaCl (conditions of Fig. 3C) about 10% of the bases of denatured calf thymus DNA are in interbase hydrogen-bonded, base-stacked hairpin structures. Fig. 3 shows that in the presence of almost (50 to 80%) saturating concentrations of gene 32-protein, the hyperchromic change in ultraviolet absorbance with temperature characteristic of the uncomplexed polynucleotides is largely abolished, in keeping with the notion that gene 32-protein binding at room temperature fully unstacks the bases.

In Fig. 3, the increase in optical density with temperature (at 260 nm) of the polynucleotide solutions, with and without gene 32-protein, is calculated relative to the optical density of the control at 24°C; the absorbance of gene 32-protein alone at 24°C has been subtracted from the absorbance of the complex. Between 50° and 60°C, gene 32-protein itself is heat-denatured, with a concomitant increase in A260. Beyond this protein transition the slope of the complex melting is seen to parallel the control curve in all three of the melting experiments. This indicates that either denatured protein does not interact with single strands, or that it interacts without greatly perturbing the remaining stacking interactions.

Thus, we conclude that at temperatures below ~50°C native gene 32-protein induces a pronounced increase in the hyperchromicity of the single strands. In the denatured DNA sample, as well as in poly[d(A-T)], this increase results both from the loss of stacking interactions in double helical intrachain hydrogen-bonded sequences and from a decrease in the optically detectable residual interactions between the bases along the single strand. In poly(dA) and poly(rA), the binding difference spectra for the gene 32-protein-poly(dA) complex systems of A. Each difference spectrum was calculated by subtracting the sum of the control poly(dA) spectrum and the appropriate gene 32-protein spectrum from the observed complex spectrum. Curves are numbered according to the input protein concentrations listed in the legend of A.
characterize the interaction, this observation provides assurance that gene 32-protein does bind to poly(dT) under the conditions of the experiment.

Circular Dichroic Spectra of Single-stranded Polynucleotides Complexed with Gene 32-Protein—CD spectra for poly(dA), poly(rA), poly(dT), and heat-denatured and recooled calf thymus DNA, each protein-free or complexed with one or more concentrations of gene 32-protein, are shown in Fig. 4, A to D. The contributions of gene 32-protein itself to the CD spectra have not been subtracted, but it has been shown (19) that at these concentrations the spectral contributions of the protein are negligible at wavelengths longer than 245 nm.

Inspection of Fig. 4, A to D shows that the general effect of gene 32-protein binding is a reduction in the intensity of the polynucleotide CD spectra, decreasing, in particular, the intensity of the longer wavelength positive bands. This is (qualitatively) the expected effect if complex formation uncouples the transition moments of the vicinal bases, as suggested by the effects on the ultraviolet absorption spectra cited above. However, the CD results are not simply comparable to spectral changes induced by heating alone. In Fig. 4A we compare CD spectra of poly(dA) (at 24°) in the presence of increasing concentrations of gene 32-protein, with a sample of free poly(dA) heated to 45°. The intensity of the positive band centered at 262 nm is strongly suppressed by gene 32-protein binding (curves 2 and 3, Fig. 4A), while this band is somewhat enhanced by thermal perturbation (curve 4, Fig. 4A). The band at 280 nm is less affected by complex formation, as is the negative band at 250 nm. Partially similar behavior is seen for poly(rA) complexed with gene 32-protein (Fig. 4B) in that again an appreciable decrease in the intensity of the positive (long wavelength) band is seen. In poly(rA), however, the negative band also loses intensity on complex formation.

The CD spectra of dA and rA mononucleotides are very similar to one another, showing a negative band at 264 nm and a positive band around 220 nm (26). Neither mononucleotide spectrum resembles that of the homologous unliganded polymer (Fig. 4, A and B) and the spectra induced in the polymers as a consequence of gene 32-protein binding also do not resemble those of the monomers.

of gene 32-protein disrupts the single-stranded stacking relationships characteristic of these polymers.

Base stacking is not believed to occur in poly(dT) (29); thus, a solution of this polynucleotide shows little optical density change at 260 nm on heating. There is also little change in the optical density of poly(dT) on combination with gene 32-protein (data not shown), confirming that the major source of gene 32-protein-induced ultraviolet hyperchromicity in the other polymers is indeed a decrease in base stacking. The circular dichroic absorption studies described below (Fig. 4C) indicate that gene 32-protein does induce a change in the secondary structure of poly(dT), although one which is not detectable in the ultraviolet absorption spectrum. In addition to helping to
As pointed out above, the CD spectrum of poly(dT) (Fig. 4C) is altered on gene 32-protein binding, even though no changes are observed in the ultraviolet absorption spectrum. The complexed spectra are similar to those observed for the poly(rA) - gene 32-protein complex, in that both positive and negative bands lose intensity. There is no shift in the wavelength of crossover for poly(dT), however, while there is a shift in the poly(rA) crossover point. This may be due to the apparent shift to longer wavelengths of the poly(rA) negative band upon complex formation and could be a consequence of base unstacking in this polymer on gene 32-protein binding.

A shift in the CD crossover point towards longer wavelengths is also seen when denatured calf thymus DNA is complexed with gene 32-protein (Fig. 4D). The observed CD spectrum of heat-denatured and recooled calf thymus DNA (curve 2) is identical to that originally described by Brahms and Mommaerts (30) at room temperature and under similar solvent conditions. When their sample as heated to 80° the CD positive band was seen to lose intensity, but not to as great an extent as observed isothermally here in the gene 32-protein complex. Furthermore, when heated to 80° the crossover point for calf thymus DNA occurs at 258 nm and remains there while the sample is recooled (30). In contrast, gene 32-protein binding shifts this crossover point markedly to longer wavelengths (Fig. 4D).

The CD spectra of polynucleotides complexed to gene 32-protein do not resemble published spectra obtained with uncomplexed polynucleotides. However, they do show considerable similarity to CD spectra of gene b-protein of fd bacteriophage complexed to single-stranded fd DNA, poly[d(A-T)], and poly(rA) (31, 32). Day (31) and Anderson et al. (32) also interpreted these spectra in terms of uncoupling of the transition moments of vicinal bases as a consequence of complex formation and showed that gene 5-protein binding considerably increases the ultraviolet hyperchromism of the polynucleotides.

Substantial changes in polynucleotide CD spectrum are also observed when gene 32-protein forms a complex with double helical poly[d(A-T)] (Fig. 5A). The input ratios of polynucleotide and gene 32-protein which produce the CD spectra shown in this figure (curves 2, 3, and 4) are nearly equivalent to those used in the melting experiments shown in Fig. 7A (curves 3, 4, and 5; further discussed below) and result in from about 30 to 65% denaturation of the helical structure. The control spectra of double helical and thermally denatured poly[d(A-T)] are shown in Fig. 5B where it is seen that heating results in a shift of the strongest positive band from 261 to 270 nm.

Inspection of the spectra of poly[d(A-T)] complexed with gene 32-protein (Fig. 5A) reveals that the bands near 270 and 280 nm are not strongly perturbed by the interaction and that the band at 261 nm is still present although markedly.

* After this work was completed, a paper appeared by Anderson and Coleman (33), in which they report CD spectra of single-stranded fd DNA complexed with gene 32-protein and with Escherichia coli binding protein, which closely resemble the gene 32-protein-perturbed polynucleotide spectra presented here. The generality of this binding protein-perturbed CD spectrum suggests that even though each of these proteins has a very different effect upon the long range conformation of single-stranded DNA as visualized by electron microscopy (as well as different specific effects on the template activity of the DNA, see ref. 34), all have similar uncoupling effects at the base-base interaction level.
that the loss of CD positive band intensity at 261 nm upon complex formation can be accounted for by the loss of double helical structure. It is noted, however, that the positive band at 270 nm is not well developed in the poly[d(A-T)]-gene 32-protein complex; the intensity is, in fact, somewhat depressed relative to calculated curve (Fig. 5A). Consistent with our observations using other polynucleotides, it is apparent that the gene 32 protein interaction results in a decrease in the intensity of the CD positive band characteristic of single-stranded poly[d(A-T)].

These studies of the optical properties of single-stranded polynucleotides show that gene 32-protein binding disrupts both the base stacking in double helical hairpin regions and interactions between bases along the single strand. These results are consistent with the proposed linear expansion of the polynucleotide single strand as a consequence of complex formation with gene 32-protein (4, 16). These experiments also indicate that the gene 32-protein-bound single strand may be held in a unique conformation, since the interactions between the base chromophores as observed by circular dichroism are not like those between stacked bases or between bases in single strand structures from which most of the secondary structure has been removed by heating. Electron microscopy reveals that in the complex there is a 4.6 Å separation between the nucleotides (18), while in a fully extended single-stranded polynucleotide base separation is ~1 Å (4). Therefore, although the nucleotides are not stacked, neither is the strand fully extended. Perhaps the regularity of continuous protein binding induces a helical sense to the complexed single strand, with a small angle of rotation per residue and many residues per turn (16). Such a conformation might account for the observed CD spectra of the complexed polynucleotides.

C. Site Size for Gene 32-Protein Binding to Single-stranded Polynucleotides

We have taken advantage of the ultraviolet hyperchromism due to the base unstacking induced by gene 32-protein on binding to partially stacked single-stranded polynucleotides to measure the sites size of the bound protein. Fig. 6 shows a titration curve involving a fixed amount of poly(dA) (0.05 M Na+, pH 7.7) to which we add successive aliquots of gene 32-protein. We see a linear increase in poly(dA) hyperchromicity with increasing protein, up to a nucleotide-protein ratio of ~6.7:1; beyond this point, further additions of protein have no effect. We conclude from such measurements that gene 32-protein, binding cooperatively to single-stranded DNA, covers ~6.7 nucleotide residues per 35,000 dalton monomer. This result is in reasonable accord with the site size of ~7.5 nucleotides/protein monomer inferred from melting profiles of gene 32-protein-complexed poly[d(A-T)] (see below) and the value of ~5 nucleotides/protein monomer measured by titration of the intrinsic tryptophan fluorescence of the protein with both poly(dA) and poly(rA) (18), given the very different physical bases of these techniques.

D. Ionic Strength Dependence of Binding of Gene 32-Protein to Single-stranded Polynucleotides

Experiments such as that illustrated in Fig. 6 permit us to make some qualitative statements about both the affinity of gene 32-protein binding cooperatively to a single-stranded (initially partially stacked) polynucleotide such as poly(dA), and the ionic strength dependence of this affinity. In discussing this affinity we must bear in mind that in this type of experiment the binding constant actually estimated is $K_w$, the...
dependence of the nature of the estimates and assumptions involved in the binding constant for isolated binding of gene 32-protein to a protein-free poly(dA) control after the optical density contribution of the input protein is subtracted from the observed optical density.

FIG. 6. Titration of poly(dA) hyperchromicity induced by gene 32-protein binding to determine single strand site size; 0.05 mM NaCl, 2 mM NaHPO₄, 0.1 mM Na₂EDTA, pH 7.7, 24°C. Poly(dA) concentration, 2.98 x 10⁻⁵ M. Hyperchromicity at 260 nm is determined relative to input protein concentrations ranging from 0 to 7 x 10⁻⁶ M, indicating that at this salt concentration Kᵦ > 10⁷ M⁻¹.

It is shown in the next section that Kₑ (the isolated site binding constant for gene 32-protein to a single-stranded lattice) is very ionic strength-dependent, decreasing, with increasing Na⁺ concentration, from values in excess of 10⁸ M⁻¹ at 0.010 M Na⁺. Comparisons of the findings of the next section with the data of Kelly et al. (18) on the binding of gene 32-protein to oligonucleotides at 0.10 M Na⁺, suggest that ω ≈ 10² and that this parameter is approximately ionic strength-independent. These results, and the fact that Kₑ “passes through” a value of 10² M⁻¹ between 0.3 and 0.6 M Na⁺ (say 0.5 M Na⁺), permit us to guess that if log K decreases linearly with log [Na⁺] (as it does for ribonuclease binding to native and native DNA, this paper), then b log [Na⁺] = -2; i.e. the binding constant for isolated binding of gene 32-protein to a single-stranded lattice decreases by almost 2 orders of magnitude per 10 fold increase in Na⁺ concentration. Bearing in mind the nature of the estimates and assumptions involved in arriving at this number, we note that this ionic strength dependence of K for single strand polynucleotide binding of gene 32-protein is comparable to that for binding of this protein to native DNA.

In the course of these experiments we also attempted to perform a poly(dA) titration with gene 32-protein at very low ionic strengths (~0.004 M Na⁺). Under these conditions, no interaction with poly(dA) (as manifested by base unstacking) was observed. This suggests that the active gene 32-protein conformation is unstable (i.e. the protein denatures) at very low ionic strength. Since the protein is fully active in denaturing and unstacking poly(dA-T) at 0.010 M Na⁺, this protein inactivation must become effective only at salt concentrations below this level. We noted also that this inactivation is apparently reversible, at least under some conditions. After periods in excess of 2 h at 0.004 M Na⁺, full poly(dA) binding activity could be restored by raising the Na⁺ concentration to 0.05 M. Longer term storage at low ionic strength may result in irreversible changes; thus, on storage we found that gene 32-protein displays much lower binding activity (as have Alberts and co-workers*). (Carroll, et al., (35) have also noted that gene 32-protein aggregation, which they associate with interactions involved in cooperative protein binding to single-stranded polynucleotides (however, see Ref. 18), appears to be irreversibility decreased at very low ionic strength.) This apparent instability of gene 32-protein at low ionic strength also prevented us from conducting poly[d(A-T)] and DNA melting experiments with this ligand in low ionic strength buffers in which the unperturbed stabilities of the double helices would have fallen into more convenient temperature ranges.

E. Perturbation of the Poly[d(A-T)] Melting Transition by Gene 32-Protein Binding and Estimation of Thermodynamic Parameters of Binding to Single-stranded DNA

Since gene 32-protein has an appreciably greater (cooperative) affinity for single-stranded than for double-stranded DNA and the binding site sizes for both lattices are comparable, we would expect this protein to be a very effective equilibrium destabilizer of the double helical conformation; much more effective, for example, than ribonuclease (see Fig. 8A in Ref. 1).

Fig. 7A shows melting profiles for double helical poly[d(A-T)] in 0.01 M Na⁺, in the presence of increasing concentrations of gene 32-protein, and demonstrates that this expectation is certainly qualitatively realized. (The dashed lines and points represent the real data, plotted as fraction melted, and the solid lines represent theoretical curves fitted to the experimental data as described below.) Qualitatively, the observed melting transitions show that the effect is dominated by a tight and cooperative binding of gene 32-protein to the single-stranded poly[d(A-T)] lattice, resulting in complete melting (with Tₘ < 14°C, which is the temperature at which the data points begin in the experiment summarized in Fig. 7A) of the fraction of the poly[d(A-T)] molecules totally complexed with gene 32-protein (due to cooperative protein binding), and virtually unperturbed melting of the remaining (free) poly[d(A-T)] molecules. This result should be compared with that of Fig. 11 in Ref. 1, which shows the form of the poly[d(A-T)] melting curves obtained with ribonuclease binding preferentially, but not cooperatively, to the single-stranded lattice. In such “non-cooperative” cases, the entire transition is shifted toward lower temperatures, but without much change in shape, in striking contrast to the markedly biphasic character

* B. M. Alberts, private communication.
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of the transitions seen for the partially saturated, highly "cooperative," binding system of Fig. 7A. Using the theoretical analysis developed by McGhee (36), we can fit these experimental melting profiles using various values of site size ($n_s$), (isolated) binding constant ($K_h$), and cooperativity parameter ($\omega$) for gene 32-protein binding to the native and denatured polynucleotide lattices in order to obtain the "best fit" values of these parameters.

The ordinates of Figs. 7 A, B, and C are plotted as fraction of double helix melted. We cannot use the fractional increase in hyperchromicity directly to represent this parameter, since (see above) gene 32-protein binding increases the hyperchromicity of the single-stranded polynucleotide above the fully melted value. Instead, we measure the fraction of the poly[d(A-T)] which is uncomplexed by determining what fraction of the total transition is represented by the second (unperturbed) phase of the biphasic melting profile in the presence of gene 32-protein. The complexed fraction is then determined by difference, and the normalized transition plotted as fraction melted, as in Fig. 7A to C. We note that this analysis is only possible at this low ionic strength because the entire poly[d(A-T)] transition goes to completion prior to the onset of thermally induced protein denaturation, which (as shown in Fig. 3 above) also increases the optical density of the sample due to increases in the extinction coefficients of the aromatic amino acids upon denaturation.

These normalized transitions are fitted, using the approach of McGhee (36), with various values of $n_s$, $K_h$, $\omega$, $n_p$, $K_p$, $\omega_p$ (the subscript h indicates parameters applying to binding to the double helix, while the subscript c designates parameters relevant to binding to the single-stranded form of the polynucleotide), and $\Delta H$ for ligand binding to each form of the polynucleotide, as well as the various parameters characterizing the helix-coil transition of the uncomplexed poly[d(A-T)]. The final parameters chosen are summarized in the legend of Fig. 7A, as well as in the detailed justifications presented for each below. We see that the final fit of theory to data (calculated in the constant total protein mode, see Ref. 36) is very good and that we are able to use this approach to define, within rather narrow limits, $n_s$, $K_h$, and $\omega$. We should re-emphasize, as stated in Jensen and von Hippel (1), that although many parameters are used here, the system is very sensitive to small changes in those we define by these measurements and thus we expect the "best fit" values to closely represent those which actually apply under the conditions of the experiment.

**Helix Coil Transition Parameters for Poly[d(A-T)]**

As helix-coil transition parameters for the melting of poly [d(A-T)] we use (as in Ref. 1) an enthalpy change ($\Delta H$) of 8.0 kcal/base pair, a helix-coil cooperativity parameter ($\sigma$) of $5 \times 10^{-4}$ (this lumped parameter includes all contributions to the cooperativity of the transition, including loop-weighting functions, etc.), and the experimentally determined midpoint of the control transition in the relevant buffer as $T_m (41.4^\circ \text{C} in the calculations of Fig. 7 A to C). $T_m$ and $\Delta H$ together establish the other helix-coil transition parameter, $s$ (the equilibrium constant for adding one base pair to a stacked sequence), as well as the dependence of $s$ on temperature. $\sigma$ is taken as temperature-independent. For further details and justification of these values and relevant literature references, see McGhee (36).

The gene 32-protein binding parameters which will

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*For a detailed theoretical analysis of the changes in shapes of melting transitions in the presence of "melting" ligands, as binding of the ligands becomes cooperative, see McGhee (36).
emerge from these calculations are quite insensitive to small changes in the values of $\Delta H$ and $\varepsilon$.

**Determination of Site Size ($n_c$)**—At high values of $K_{\text{w}}$, essentially all of the gene 32-protein present in the system is bound to coil sequences of poly[d(A-T)] which have been melted at temperatures below the experimental range (less than $14^\circ$ in the experiment depicted in Fig. 7A). Thus, under these conditions we see no helix binding of gene 32-protein and, because of cooperativity, little or no transfer of protein to coil regions which might be increasingly revealed as the temperature is increased. Thus, a virtually flat plateau is observed until the uncomplexed poly[d(A-T)] melts at essentially the temperature characteristic of the melting of the unperturbed species.

Since virtually all the protein is bound continuously to the single-stranded poly[d(A-T)] sequences, the height of the plateau region in terms of fraction poly[d(A-T)] melted becomes a very sensitive measure of binding site size. Initial trial values indicated that the plateau regions of the experimental curves in Fig. 7A are best described using value of $n_c$ of 7.5 (+0.3) nucleotide residues/protein monomer. The sensitivity of the calculations to this parameter is demonstrated in Fig. 7B, in which experimental curve 4 of Fig. 7A has been refitted using optimal parameters (see below) except using $n_c = 5$ or 10 nucleotide residues/gene 32-protein molecule. As indicated in section C, this value of $n_c$ is in reasonable accord with those obtained by base unstacking ($n_c \approx 6.7$) and by protein fluorescence quenching ($n_c \approx 5$), considering the major differences in approach represented by these measurements.

Part of the gene 32-Protein Binding to Native (Double Helical) Poly[d(A-T)]—In the calculations relating to Fig. 7, A to C, we have used a value of $K_c = 1.3 \times 10^5$ M$^{-1}$ as the binding constant for gene 32-protein to double helical poly[d(A-T)]. A value of $n_c = 10$ bases (5 base pairs) per protein monomer as the binding site size, and a value of $\omega = 1$ as the protein interaction cooperativity parameter. The values of $n_c$ and $\omega$ are those determined in Sec. A and the value of $K_c$ used is obtained by linear extrapolation of the $K_c$ values of section A on a log $K_c$ versus log $[\text{Na}^+]$ plot. Since in this phase we are fitting only the data in the plateau region and at higher temperatures, the fit of the theoretical curves to the experimental ones turns out to be independent of the choice of parameters for the binding of protein to the double helical lattice, indicating, as previously inferred, that in these parts of the transition essentially all the protein is bound to single-stranded regions. Of course, at higher salt concentrations (see below), where the first “phase” of the experimental curves we conclude that $K_c > 10^5$ M$^{-1}$ in 0.01 M Na$^+$ (at $\omega = 10^3$, see below). We note that a $K_c$ of $\sim 10^3$ at this ionic strength is in good accord with the binding constant of $\sim 10^5$ M$^{-1}$ obtained in 0.1 M Na$^+$ for the di-nucleotide model compounds which exhibit the total free energy of the isolated (non-cooperative) binding interaction (see Ref. 18) and the estimate of an approximately 2 orders of magnitude decrease in $K_c$ with a 10-fold increase in sodium ion concentration (see section D, above).

**Determination of Binding Constant ($K_c$) to Single-stranded Lattice**—The best value of $K_c$, for the conditions of Fig. 7, A to C, was determined as shown in Fig. 7B. Using values of $n_c = 7.5$ nucleotide residues/protein monomer (see “Determination of Site Size ($n_c$)” and $\omega = 10^3$ (see below), we have calculated binding curves for values of $K_c$ ranging from $10^5$ to $10^8$ M$^{-1}$. As Fig. 7B clearly shows, the calculations for $K_c = 10^3$ and $10^4$ M$^{-1}$ do not generate theoretical curves with plateau regions extending down to $14^\circ$ (the lowest temperature at which experimental data were obtained). Thus, in the absence of data in the first “phase” of the experimental curve we conclude that $K_c > 10^4$ M$^{-1}$ in 0.01 M Na$^+$ (at $\omega = 10^3$, see below). We note that a $K_c$ of $\sim 10^3$ at this ionic strength is in good accord with the binding constant of $\sim 10^5$ M$^{-1}$ obtained in 0.1 M Na$^+$ for the di-nucleotide model compounds which exhibit the total free energy of the isolated (non-cooperative) binding interaction (see Ref. 18) and the estimate of an approximately 2 orders of magnitude decrease in $K_c$ with a 10-fold increase in sodium ion concentration (see section D, above).

Effect of Gene 32-Protein Binding on Poly[d(A-T)] Melting Transitions at Higher Ionic Strengths—Melting transitions of poly[d(A-T)] in the presence of gene 32-protein were investigated at higher ionic strengths. Such transitions at 0.05, 0.10, and 0.20 M Na$^+$ (in the same buffer used in the experiments of Fig. 7, A to C) are shown in Fig. 8, A and B. Clearly, the situation is more complex and the sum of these complications makes it virtually impossible to subject these transitions to a quantitative analysis of the sort employed with Fig. 7, A to C. As the sodium ion concentration is increased the $T_m$ of poly[d(A-T)] shifts to higher temperatures. The temperature-induced native → denatured transition of gene 32-protein, however, is apparently less salt-dependent. Thus, in contrast with the melting experiments depicted in Fig. 7A, the protein melting transition occurs before or during the uncomplexed poly[d(A-T)] helix-coil transition at higher ionic strengths (Fig. 8, A and B). The resulting “roller-coaster” transitions are remi...
suggesting that the cooperativity factor, \( \epsilon \), is probably no
dependence, tending toward 10 in high salt. The plateau region
perturbed poly[d(A-T)] transition is seen to be rather broad,
to note that denatured gene 32-protein provides some poly-
the data shown in Fig. 8. (a) The initial phase of the protein-
so dominant in the low salt experiments (Fig. 7A) may just be
appearing, in these high salt experiments, as protein denatu-
ration commences. (b) The temperatures at which the initial
(ligand-complexed) phase of the poly[d(A-T)] melting profile
occurs permit us to estimate that \( K_a \) in 0.2 M Na\(^+\) is indeed,
as expected from the train of reasoning outlined above, some-
where near \( 10^{10} \) M\(^{-1}\) if \( \omega \approx 10^3 \) and double helical binding is of
about the expected magnitude.

Thus, in summary, while a quantitative description of these
melting profiles is precluded, the experimental transitions do
show that gene 32-protein can bring about equilibrium melting of
poly[d(A-T)] under these conditions as well. These experi-
ments also provide semiquantitative support for the param-
ters estimated in the helix-coil transitions measured at 0.01 M
Na\(^+\) and indicate that our estimates of the ionic strength de-
pendencies of these parameters are reasonable.

**F. Gene 32-Protein Does Not Destabilize Native DNA**

Since gene 32-protein has a (cooperative) affinity constant
\( K_{a \omega} \) for single-stranded DNA which can be as much as 4 to
5 orders of magnitude greater than its affinity for the native
(double helical) structure, we should be able to use this pro-
tein to very effectively destabilize the native DNA double
helix. The melting effect of this ligand on hairpin loops in
denatured and recoiled calf thymus DNA and on double helical
poly[d(A-T)] reinforces this expectation, as does the
marked destabilizing effect observed with the much "weaker"
melting protein, ribonuclease, on native DNA (1). Based
on these facts, at \( \sim \) 0.01 M Na\(^+\), saturating quantities of gene
32-protein should lower the melting transition of native DNA
by at least 50\(^\circ\). Yet, Alberts and Frey (4) had previously re-
ported that gene 32-protein does not melt native DNA, and in
an exhaustive series of experiments we have confirmed that
gene 32-protein has no apparent effect on melting transitions of
a series of different natural DNAs. Fig. 9A shows a melting curve for native T7 DNA in the
presence and absence of saturating quantities of gene 32-protein
at 0.01 M Na\(^+\) (pH 7.7). As this plot shows, gene 32-protein
has no destabilizing effect on T7 DNA under these conditions,
which are identical to the conditions which apply to Fig. 7, A
to C, in which enormous destabilization of poly[d(A-T)]
melting was seen. The arrow in Fig. 9A indicates the temperature
at which one such experiment was held for 8 h in an effort to
approach equilibrium. The melting transition starting at about
50\(^\circ\) in Fig. 9G, A and B is that of protein. The DNA melting
transition in the presence of denatured gene 32-protein is a
consequence of a polycationic stabilization of the native DNA
by the denatured protein at low ionic strength, very similar
to the stabilization of native DNA by denatured ribonuclease
seen in the preceding paper (1). The dotted line in Fig. 9A
indicates the expected gene 32-protein-perturbed T7 melting
curve for this situation, based on the same thermodynamic
parameters used in the calculations of Fig. 7A.

Clearly, equilibrium is not attained in these transitions when
approached from the low temperature side, i.e. the expected
equilibrium destabilization is prevented by a kinetic block.
The nature of this block may be guessed at when one recalls
that isolated binding of gene 32-protein to single strand DNA
is less than 2 orders of magnitude stronger than binding to
the double-stranded polynucleotide lattice, and furthermore
that cooperative binding to single strands appears to result in
appreciable (presumably energy-requiring) deformation of
the polynucleotide backbone. Thus, we expect that relatively large
melted sequences (loops) are required for nucleating the

\[ \text{Relative Hyperchromicity} \]

\[ \text{Temperature (°C)} \]

\[ \text{Relative Hyperchromicity} \]

\[ \text{Temperature (°C)} \]

![Graph](image.png)

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*Although gene 32-protein shows a net negative charge at neutral pH, the occurrence of this polycationic stabilization by the denatured (presumably random coil) protein suggests that portions of the chain containing positive charges are preferentially bound under the conditions of these experiments, with negatively charged sequences presum-
ably "dangling free."*
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T7 DNA 32-Protein

0.012 M Nat

A.

Sonicofed T7 DNA: 32-Protein

B.

Effective Melting Cannot Be Nucleated at Ends of DNA Molecules—In order to determine whether sufficient melting (“fraying”) occurs at the ends of native DNA to nucleate the gene 32-protein-driven destabilization, we extensively sonicated native T7 DNA. The sedimentation coefficient was decreased from 28.1 S to 7.0 S in this treatment, indicating, via the molecular weight versus sedimentation coefficient calibration of Eigner and Doty (37), that the number of molecular ends is increased ~100-fold. Fig. 9B shows a melting profile for this sonicated DNA, in the presence and absence of gene 32-protein. The results are virtually identical to those obtained with whole T7 DNA (Fig. 9A), indicating that no detectable nucleation occurs at the ends of the native DNA molecule under these conditions. This finding is in good agreement with a recent electron microscopic examination of DNA subjected to partial denaturation by formaldehyde, which also indicated virtually no melting from the ends, as well as experiments involving ribonuclease destabilization of sonicated DNA (1). Thus, at temperatures approximately 20° below the Tm (as in Fig. 9B), native DNA apparently does not “fray” sufficiently from the ends to expose the number of single-stranded nucleotide residues (10 to 200) required to nucleate gene 32-induced destabilization of the DNA molecule.

Kinetic Block to Native DNA Destabilization Is Not Dependent on Base Composition—The reversible denaturation of gene 32-protein induced by low ionic strength prevents us from going below approximately 0.008 M Na+ in experiments such as those of Fig. 9, A and B. Thus, we are unable to get to within less than 15°–20° of the Tm of the unperturbed T7 native DNA structure prior to the onset of protein denaturation. This is already amply close to Tm to demonstrate the existence of the kinetic block to destabilization, since calculations (see above) indicate that at all temperatures above freezing under these conditions we are well within the region of the DNA phase diagram in which equilibrium favors the melting of the double helical form of the DNA as a consequence of gene 32-protein complex formation with single-stranded sequences. However, it would be important to know how close to Tm one needs to come before the kinetic block is overcome (at a reasonable rate) and equilibrium attained. In order to vary the melting temperature of the DNA (at constant Na+ concentration), we therefore examined the melting behavior of sonicated and unsonicated calf thymus DNA (58% dA·dT base pairs), Clostridium perfringens DNA (69% dA·dT), and Micrococcus lysodeikticus DNA (28% dA·dT), as well as the T7 DNA (52% dA·dT) considered above. The melting experiments were carried out at 0.010 M Na+, and in all cases essentially no DNA melting was observed at temperatures below the denaturation temperature of the gene 32-protein (data not shown), although...
for all systems, if equilibrium had been reached, melting would have been complete at 0–25°. 10, 11

**Discussion**

By definition (see Introduction of Ref. 1), a polynucleotide melting protein is one which, under a given set of conditions, is able to achieve a higher binding density on a single-stranded than on a double helical structure. This binding characteristic brings about a net stabilization of the single-stranded, relative to the double helical, conformation; the equilibrium end result is destabilization of the double helical structure manifested by a shift of the melting temperature (Tm), and of the entire melting profile, to lower temperatures. In effect, the formation of the single-stranded polynucleotide–melting protein complex competes with double helix formation for single-stranded sequences and thus lowers the chemical potential of the single-stranded form of the polynucleotide.

The magnitude of the melting temperature shift induced by melting protein binding, as well as the overall shape of the melting transition, will depend on the relative magnitudes of the binding constant for the protein to the helix and coil forms of the polynucleotide (when binding site sizes are comparable) as well as on the free protein concentration (activity) and on whether the binding is cooperative in protein concentration. Theoretical treatments of the binding of melting protein like ligands to lattices of the polynucleotide type have been presented by McGhee and von Hippel (13) and Schellman (38), and extended to take into account the effects of such binding on helix ↔ coil equilibria by McGhee (39) (see these references for citations to the extensive earlier theoretical literature). In the preceding paper (1), the thermodynamics of ribonuclease complex formation with various conformations of DNA was explored, and the consequences of the (non-cooperative) preferential binding of this protein to single-stranded DNA were shown to follow the predictions of theory reasonably well.

As described under “Results,” the preferential cooperative binding of T4-coded gene 32-protein to single-stranded regions of the synthetic polynucleotide poly[d(A-T)] also has theoretically predictable consequences for the equilibrium melting behavior of this double helical structure. Binding constant measurements suggested that this protein should destabilize double helical DNA to a much greater extent than ribonuclease, and these expectations are qualitatively and quantitatively borne out for the ligand perturbed poly[d(A-T)] melting results. In fact, as detailed above, we have utilized this accord with theory to estimate denatured DNA binding constants and cooperativity parameters by comparing the experimental melting profiles (in regions of the phase diagram where the free energy of binding to single-stranded sequences greatly exceeds the free energy of helix stabilization, and protein is limiting) with computer-simulated theoretical melts, and obtained results in close agreement with experimental estimates based on other approaches (see “Results,” and Ref. 18). All these measurements have yielded an estimated cooperativity parameter (ω, defined as the equilibrium constant for the translocation of a binding protein molecule from an isolated to a contiguous binding site) of ∼102; and a cooperative binding parameter (essentially Kω, where K is the intrinsic binding constant for a single protein to an isolated lattice site) of ∼10 M-1 in ∼0.1 M Na+. At this ionic strength the binding constant for gene 32-protein to double helical DNA is appreciably less than 10 M-1; also, binding to native DNA is non-cooperative.

The melting experiments with natural double helical DNAs, which indicate that gene 32-protein does not lower the melting temperature of these structures, appear anomalous and at odds with the above results. From a thermodynamic viewpoint (based on measured values of Kω, ω, n, Kω, ω, and na), we can only conclude that equilibrium is not attained for these systems when approached from the low temperature direction, and thus that a double helical DNA molecule, in the presence of quantities of gene 32-protein adequate to saturate the coil form at 15–20° below the temperature of the unperturbed melt, represents a metastable structure (i.e., a nonequilibrium state). Structurally this result, in contrast to the destabilization of native DNA by the non-cooperative binding of ribonuclease, requires that the formation of a stable gene 32-protein–single-strand complex within a natural, primarily double helical DNA molecule be a very unlikely event. Our findings on the nature of the gene 32-protein–single-stranded DNA complex provide support for this notion. Thus we have shown, by a combination of ultraviolet absorbance and circular dichroic spectroscopy, that the cooperative binding of gene 32-protein molecules to single-stranded polynucleotides puts the structure into a highly distorted conformation in which the transition moments of neighboring bases are significantly decoupled. The electron microscopic results of Delius and co-workers (16) are in accord with this interpretation, in that they show that the DNA chain is appreciably extended (to ∼4.6 Å/nucleotide) from the equilibrium single-stranded backbone structure, which does not differ much from that assumed by each individual strand in solution in the classical B conformation (∼3.4 Å rise/nucleotide). In contrast (1), the binding of ribonuclease to single-stranded DNA does not appear to deform the DNA conformation appreciably from the conformation assumed by the single-stranded polynucleotide chain in the absence of the protein ligand.

Thus, we suggest that single-stranded loops of a size required to accommodate ribonuclease appear spontaneously (by “breathing” processes; i.e., structural fluctuations) in native DNA at temperatures close to the melting transition and with sufficient frequency to permit ligand binding equilibrium to be attained. On the other hand, gene 32-protein requires a much larger loop to permit stable binding since (a) this protein is larger than ribonuclease; (b) it must distort the polynucleotide backbone appreciably to permit stable binding; and (c) cooperative (contiguous) binding may be necessary to permit the
attainment of an adequate free energy of binding per protein monomer, both because a distribution over several bound protein monomers of the free energy of distortion of the backbone may be required and because contiguous binding may be needed to induce a conformational change in the protein which is necessary for tight binding to the polynucleotide lattice (18). It appears that loops of adequate size to permit stable gene 32-protein binding are not formed with significant frequencies at temperatures 15° or more below the unperturbed T_m of natural DNA. In addition, it appears that single-stranded sequences of sufficient length to permit effective initiation of cooperative gene 32-protein binding are also not exposed in natural DNA under these conditions by fraying of the double helix from the ends of the molecule, or from single-stranded “nicks” which may be present at random along the double helix.

On the other hand, since gene 32-protein binding equilibrium is apparently achieved in poly[d(A-T)] experiments, we conclude that the transiently formed hairpin or palindromic hydrogen-bonded regions which can occur in this structure because of its special nucleotide sequence do provide nucleation sites adequate to initiate gene 32-protein single strand binding at temperatures far below the unperturbed T_m of this polynucleotide.

These structural and thermodynamic considerations are also relevant to the in vivo role of gene 32-protein in T4 DNA manipulation. The observed kinetic block to native DNA melting by gene 32-protein can serve to protect the native DNA against melting as a consequence of transient fluctuations in protein concentration or cellular environment. Since the exact ionic environment of the cell is unknown, we cannot calculate quantitatively the equilibrium T_m of the DNA in the cell in the presence of physiological gene 32-protein concentrations. However, we surmise that the DNA double helix must represent the species stable at equilibrium at these gene 32-protein concentrations, since, as a consequence of replication, recombination, and perhaps transcription, every DNA sequence must pass through an intermediate single-stranded state several times during the lytic T4 phage infection cycle. Assuming that the double helical conformation is the stable form of DNA in vivo, the tight coupling of gene 32-protein synthesis to the amount of single-stranded DNA present in the cell (see discussion in the accompanying papers by Gold and co-workers (39, 40)) is doubtless critically involved in preventing a potentially lethal overproduction of gene 32-protein.

The in vitro properties of gene 32-protein are consistent with current views of its physiological (in vivo) function, which may include protecting single-stranded sequences of DNA (and perhaps in RNA) against nuclease attack and facilitating double helical DNA renaturation by destabilizing metastable “hairpins” of fortuitously complementary sequences which can form within single-stranded regions during various “base pair matching” processes in replication and recombination. For a further consideration of these in vivo effects, as well as of the consequences of the in vitro properties of gene 32-protein for possible autoregulation of its own biosynthesis, see Ref. 18 and the papers of Gold and co-workers (39, 40) which follow.

Acknowledgments—We are grateful to Dr. James McGhee for many helpful and stimulating discussions of nucleic acid helix ↔ coil transitions in the presence of binding ligands and for his extensive assistance in applying his theoretical approaches to the results presented here. We are also grateful to Mrs. Ying Huang for isolating and purifying some of the gene 32-protein preparations used in these studies.

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