Phosphorus-31 and Fluorine-19 Nuclear Magnetic Resonance of Gene 5 Protein–Oligonucleotide Complexes†

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ABSTRACT: Binding of several 5′-phosphate mono-, di-, and oligonucleotides to gene 5 protein of bacteriophage fd has been examined by ^{31}P NMR. The diester phosphate resonances of $d(pA)_4$ and $d(pA)_8$ shift upfield on protein binding and show identical spectra consisting of three separate resonances, indicating three unique diester conformations common to both complexes. The chemical shifts of the bound diester phosphates, −1.10 to −1.33 ppm, suggest that the phosphodiester backbone of the bound nucleotide remains in a relatively extended gauche–trans configuration. The ^{31}P resonance of the 5′-phosphate on nucleotides one to eight residues in length shifts upfield by 0.6–1.6 ppm on binding to gene 5 protein. This upfield shift reflects a specific binding site for the 5′-phosphate dianion. Even mononucleotides are directed to this site and bind in an approximate 1:1 molar ratio, despite the fact that four base-binding pockets must exist on each gene 5 monomer. Both ^{31}P and ^{19}F NMR (of a fluoronated mononucleotide) detect a minor binding mode which may reflect weaker binding of mono- and dinucleotides to these other sites. The ^{31}P NMR spectra of the protein-bound diester phosphates change substantially on removal of the 5′-terminal dianion, confirming the prominent role of the dianion in oligonucleotide binding. The conformation of the bound 5′-oligonucleotides may thus differ in some respects from the nucleotide residues in polynucleotide or DNA complexes of gene 5 protein. ^{31}P and ^{19}F NMR of 5-fluoro-2′-deoxyuridine 5′-phosphate bound to gene 5 protein shows that this mononucleotide binds to the 5′-phosphate site in the same manner as other mononucleotides. The ^{19}F nucleus at the 5 position of the base undergoes a 0.6-ppm upfield shift, compatible with base–aromatic ring stacking previously suggested by ^{1}H NMR studies of the tyrosyl and phenylalanyl ring protons of the protein. The T_1 of the ^{19}F increases from 2.6 s in the free nucleotide to 6.6 s in the bound form, suggesting a hydrophobic base-binding pocket.

Gene 5 protein is a phage-coded, single-stranded DNA-binding protein produced following infection of *Escherichia coli* with the filamentous bacteriophage fd [see Coleman & Oakley (1980) for a review]. A functional gene 5 protein is essential for replication of the bacteriophage and has been shown to be required to shift phage-directed DNA synthesis from the double-stranded replicative form to the single-stranded viral form (Salstrom & Pratt, 1971; Mazur & Model, 1973). Although the gene 5 protein has a greater affinity for adenine- as opposed to thymine-containing regions, it will bind to single-stranded DNA of any sequence and has been shown to bind in a highly cooperative manner (Alberts et al., 1972; Coleman et al., 1976).

The gene 5 protein (M_r 9689) is composed of 87 amino acids whose sequence has been determined (Nakashima et al., 1974). A variety of spectrostopic studies have shown that the gene 5 monomer interacts with four nucleotide bases along the DNA chain (Alberts et al., 1972; Pratt et al., 1974; Anderson et al., 1975). Extensive ^{1}H and ^{19}F NMR investigations of the mode of binding of nucleotides to gene 5 protein have taken advantage of the binding of the protein to tetra- and octanucleotides [$d(pA)_4$, $d(pT)_4$, $d(pA)_8$, $d(pT)_8$], and these have served as model nucleotide complexes (Coleman et al., 1976; Garssen et al., 1977; Coleman & Armitage, 1978; Alma et al., 1981). Proton NMR studies of the aromatic region of the protein and its oligonucleotide complexes at frequencies of 270, 360, and 500 MHz all show at least two tyrosyl residues and one phenylalanyl residue to interact with oligonucleotides (Hilbers et al., 1978; Coleman & Armitage, 1978). Chemical modification of gene 5 protein–polynucleotide complexes has shown that three of the five tyrosyl residues of the protein appear to be involved in nucleotide binding (Anderson et al., 1975).

On the basis of the observation of base-dependent, nucleus-independent, upfield chemical shifts (greater for purine than pyrimidine oligonucleotides) of the tyrosyl (^{1}H and ^{19}F) and phenylalanyl (^{1}H) ring nuclei accompanying complex formation, a model of the nucleotide binding groove has been proposed in which the bases intercalate with the aromatic rings (Coleman & Armitage, 1978). A study reported here with a ^{19}F-labeled mononucleotide shows that the ^{19}F nucleus on the base undergoes a similar upfield shift on binding to gene 5 protein.

The long rotational correlation times associated with the polynucleotide complexes of gene 5 protein have precluded high-resolution NMR studies of the more natural polynucleotide complexes, although it has been tacitly assumed that tetra- and octanucleotide complexes represented most of the facets of polynucleotide binding. ^{31}P is an obvious third nucleus with which to assess binding modes by NMR methods, but relatively small changes in chemical shift and the lack of a firm basis for interpreting ^{31}P chemical shifts have limited the usefulness of ^{31}P NMR. Hence, only a few isolated ^{31}P NMR spectra of oligonucleotides bound to gene 5 protein have been reported (Coleman et al., 1976; Garssen et al., 1977). In the course of a more precise determination of the changes in chemical shift of the ^{31}P nuclei of an oligonucleotide bound to gene 5 protein, we have discovered that the "unnatural" 5′-phosphate present on the oligonucleotides does significantly alter the mode of binding. The 5′-phosphate is essential for formation of a rigid oligonucleotide complex in which a uniform binding mode is established. While many spectroscopic studies are limited to the oligonucleotide complexes, these valuable models may not mimic in all details the binding modes of polynucleotide complexes. The major features of

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the ^{31}P NMR results on oligonucleotide complexes of gene 5 protein are summarized here.

Materials and Methods

Gene 5 Protein. Homogeneous gene 5 protein was prepared by DNA–cellulose chromatography as previously described (Alberts & Herrick, 1971; Anderson et al., 1975). Protein concentration was determined by using $E^{0.1\%}_{276nm} = 0.72$.

Nucleotides. 5-Fluoro-2′-deoxyuridine 5′-phosphate (FdUMP)[1] was purchased from Sigma; all remaining nucleotides came from Collaborative Research (Waltham, MA). Nucleotides were dissolved in H_2O and extracted with a CCl_4 solution of dithizone to remove paramagnetic metals. Nucleotides were added to protein solutions by lyophilizing the appropriate amount of nucleotide from H_2O and adding protein solution. Nucleotide concentration was determined by using the absorption coefficient given by the manufacturer.

The 5′-dephosphorylated nucleotides dA_4 and dA_8 were prepared from the corresponding 5′-phosphorylated analogues by using alkaline phosphatase. The reaction mixtures were 1.5 mM in nucleotide and contained 10 mg/mL alkaline phosphatase in 25 mM Tris-HCl, pH 7.5. For the removal of enzyme and unreacted nucleotide, dA_4 was applied to a Bio-Gel P-2 column and dA_8 to a DEAE-Sephadex A-50 column.

NMR Methods. NMR spectra were recorded on a Bruker CXP-200 spectrometer. Measurements were made at 25 °C with 10-mm NMR tubes containing 2.0 mL of sample. Samples were contained in 0.025 M Tris-HCl and 90% H_2O–10% D_2O, at pH 7.5. D_2O served as the field frequency lock. pH refers to the direct reading on a Radiometer pH meter of H_2O or D_2O solutions. ^{31}P NMR spectra were determined at a spectrometer frequency of 80.9 MHz, and chemical shifts are reported in parts per million relative to 85% H_3PO_4. Digital resolution was routinely 1 data point every 2.5 Hz. ^{1}H broad-band decoupling was used throughout. ^{19}F NMR spectra were determined at a frequency of 188.2 MHz, and chemical shifts are reported relative to CF_3COOH. ^{19}F spin–lattice relaxation times (T_1) were determined by progressive saturation by using proton coupling to eliminate the substantial negative nuclear Overhauser effect (NOE). ^{19}F{^{1}H} nuclear Overhauser effects were determined by direct comparison of peak areas with and without gated proton decoupling. In the latter case, the flip angle was 90°, and the pulse repetition rate was 4.1 s.

Results

^{31}P NMR Studies of Gene 5–d(pA)$_4$ and –d(pA)$_8$ Complexes. Our previous ^{31}P NMR studies of octanucleotides [d(pT)$_8$ and d(pA)$_8$] bound to gene 5 protein were carried out at lower field (36.4 MHz) and showed small upfield shifts of the average position of the unresolved resonances of the diester phosphates to accompany binding of an octanucleotide to the protein (Coleman et al., 1976). In contrast, the present ^{31}P NMR studies at 80.9 MHz resolve resonances corresponding to individual phosphodiester bonds in the complexes. A series of ^{31}P NMR spectra for d(pA)$_4$ and d(pA)$_8$ bound to gene 5 protein are shown in Figure 1 and compared to the spectrum of free nucleotide. As in the case for all deoxynucleotides examined containing a 5′-phosphate, there is a substantial upfield shift of the resonance of the 5′-phosphate on formation of the complex with gene 5 protein. For the d(pA)$_4$ complex, this shift is ~0.6 ppm and for d(pA)$_8$ ~0.9 ppm (Figure 1B).

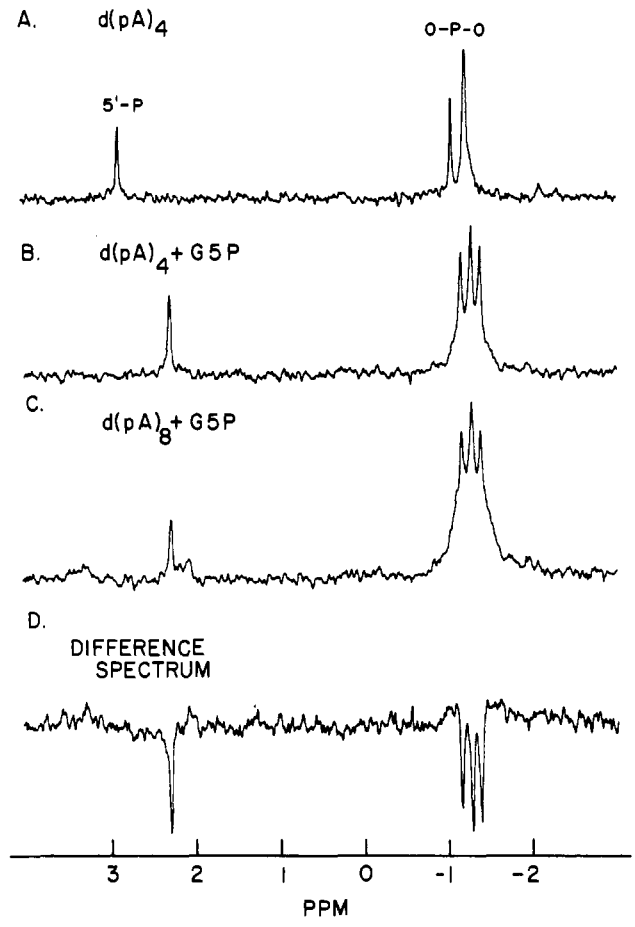

FIGURE 1: ^{31}P NMR spectra of d(pA)$_4$ (1.0×10^{-3} M) (A), d(pA)$_4$ (1.0×10^{-3} M) plus gene 5 protein (1.0×10^{-3} M) (B), and d(pA)$_8$ (0.75×10^{-3} M) plus gene 5 protein (1.5×10^{-3} M) (C); difference spectrum (D) of d(pA)$_8$–gene 5 protein minus 2.3 d(pA)$_4$–gene 5 protein. Conditions were 2.5×10^{-2} M Tris-HCl and 1.0×10^{-3} M EDTA, pH 7.5, 25 °C.

The diester phosphates of free d(pA)$_4$ show two resonances at −1.00 and −1.16 ppm, presumably reflecting some "end effects". Each of the three phosphorus atoms in phosphodiester linkage shows a separate resonance on binding to gene 5 protein, −1.10, −1.22, and −1.33 ppm. Thus, at least two and probably all three of the diester ^{31}P nuclei show significant upfield shifts from the resonances in the unbound nucleotide (Figure 1). Shifts of each diester resonance are probably 0.1–0.15 ppm, although a maximum of ~0.3 ppm could be invoked for one resonance.

In the bound state, the phosphates of the phosphodiester bonds of d(pA)$_8$ show exactly the same three resonances as observed for the d(pA)$_4$ complex, indicating very similar modes of binding (Figure 1C). A broadened underlying resonance in the case of the d(pA)$_8$ complex may be explained by the formation of some oligomeric structures (see Discussion). This finding is illustrated more graphically by the difference spectrum in Figure 1D which is formed by subtracting the ^{31}P spectrum of the d(pA)$_4$ complex from that of the d(pA)$_8$ complex by requesting the computer to normalize the amplitude of the resonance for the 5′-phosphates of the tetra- and octanucleotide in the ratio of 2.3:1.0, respectively. With the assumption of the same relaxation time for the 5′-phosphates in both complexes, this procedure compensates for the fact that each bound octanucleotide contains seven phosphodiesters instead of the six phosphodiesters present in two bound tetranucleotides. This difference spectrum shows that the spectrum of two bound d(pA)$_4$ molecules is of greater amplitude than that of the bound octanucleotide by the amount

[1] Abbreviations: FdUMP, 5-fluoro-2′-deoxyuridine 5′-phosphate; dA_4 and dA_8 (in contrast to dpA_4 and dpA_8), the tetra- and octanucleotides from which the 5′-phosphate has been removed.

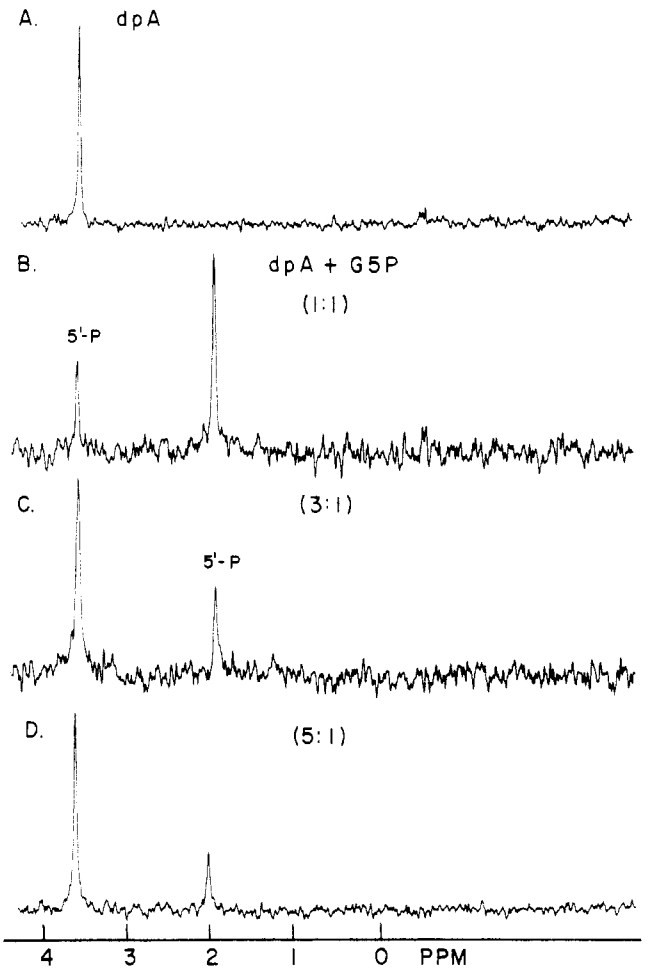

FIGURE 2: ^{31}P NMR spectra of dpA (A) in the presence of 1:1 (B), 3:1 (C), and 5:1 (D) molar ratios of nucleotide to gene 5 protein. Conditions were 0.79×10^{-3} M gene 5 protein, 2.5×10^{-2} M Tris-HCl, and 1.0×10^{-3} M EDTA, pH 7.5, 25 °C.

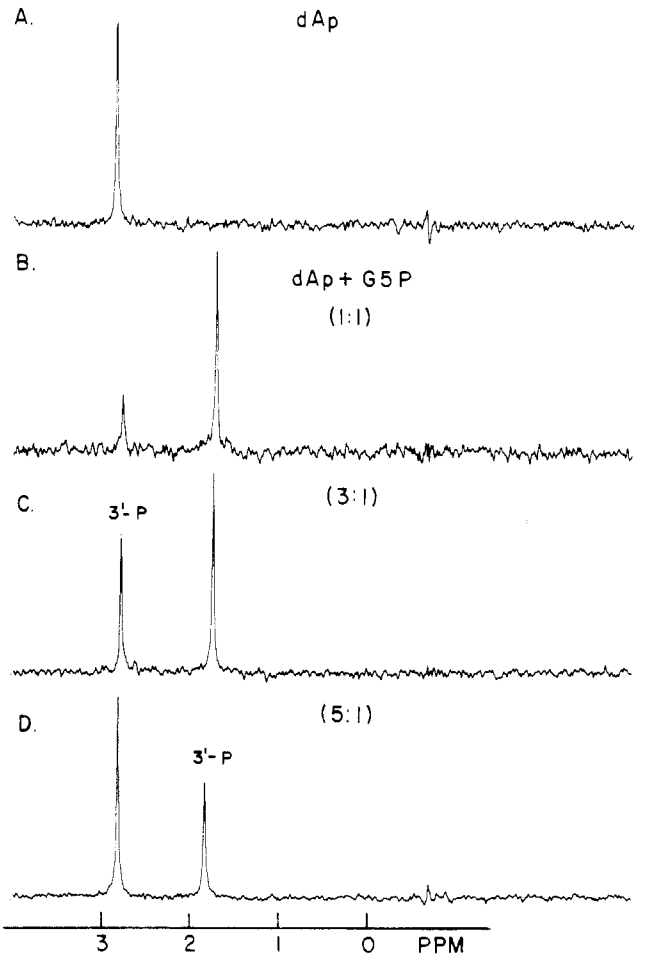

FIGURE 3: ^{31}P NMR spectra of dAp (A) in the presence of 1:1 (B), 3:1 (C), and 5:1 (D) molar ratios of nucleotide to gene 5 protein. Conditions are the same as in Figure 2.

represented by the three negative difference peaks. This resonance intensity is replaced in the octanucleotide complex by the broad positive resonance upon which the negative difference peaks are superimposed.

^{31}P NMR Studies of Mononucleotides Bound to Gene 5 Protein. While DNA-binding proteins forming stoichiometric complexes with single-stranded DNA generally cover a number of bases per protein monomer, mono- and dinucleotides which do not completely fill the binding locus have been reported to form complexes of considerable stability with gene 32 protein from T4 as determined by equilibrium dialysis and fluorescence quenching (Jensen & von Hippel, 1976; Jensen et al., 1976; McGhee, 1976; Kowalczykowski et al., 1981). In order to explore in more detail the modes of binding of nucleotides to gene 5 protein and also to explore the role of the unnatural 5′-phosphate in the binding of the model oligonucleotides to gene 5 protein, we examined the ^{31}P NMR spectra of mono- and dinucleotides with and without 5′- or 3′-phoshate groups. The sequence of ^{31}P spectra for d(pA) in the presence of 1:1, 3:1, and 5:1 molar ratios of nucleotide to gene 5 protein is shown in Figure 2. At a 1:1 mole ratio, a fraction of the d(pA) binds to gene 5 protein with the ^{31}P resonance of the 5′-phosphate shifted upfield ~1.6 ppm (Figure 2B). The ^{31}P resonance of the other fraction remains very near that expected for free nucleotide. The two forms of nucleotide represented by the two resonances are in slow exchange on the NMR chemical shift time scale ($\Delta\delta = 1.6$) and are therefore exchanging at a rate less than ~800 s^{-1}. At a 1:1 mole ratio, the binding site, defined by the upfield resonance, appears to be ~70% saturated, judging from the relative amplitude of the two signals (Figure 2A,B).

While the resonance at 3.6 ppm cannot be distinguished from that for free nucleotide, ^{31}P and ^{19}F data presented below suggest strongly that this resonance represents a second binding mode in which the bound 5′-phosphate has a chemical shift very near to that for the free nucleotide. As the d(pA) concentration is increased, the signal at 1.99 ppm remains constant in amplitude and chemical shift as the ^{31}P signal in the position of free nucleotide increases in proportion to the excess d(pA) added. If the free phosphate is at the 3′ end of the mononucleotide, an almost identical series of spectra is obtained except that the resonance of the bound 3′-phosphate shifts upfield ~1.1 ppm rather than 1.6 ppm (Figure 3).

^{31}P NMR Studies of a Dinucleotide Bound to Gene 5 Protein. When a dinucleotide carrying a 5′-phosphate, e.g., d(pApA), is mixed in a 1:1 molar ratio with gene 5 protein, a majority of the resonance corresponding to the 5′-phosphate moves upfield ~1.3–1.5 ppm,[2] not unlike the shift observed for the bound 5′-phosphate of d(pA) (Figure 4B). As in the d(pA) case, a similar proportion, ~20%, of the 5′-phosphate resonance remains near the chemical shift characteristic of the free nucleotide (Figure 4B). In the case of the dinucleotide, however, the resonance of the diester phosphorus supplies additional information. The diester resonance is split into two resonances at *−1.03* and *−1.15* ppm, both of which are upfield

[2] The exact chemical shift of the 5′-phosphate is very sensitive to small changes in pH, temperature, and ionic strength which probably explains the small shifts observed between additions.

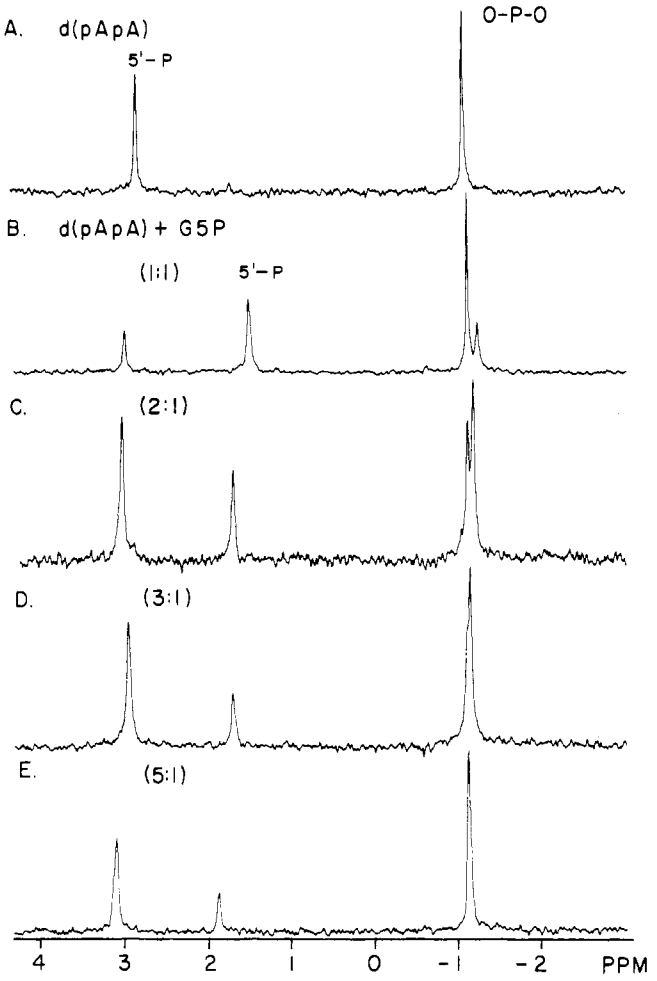

FIGURE 4: ^{31}P NMR spectra of d(pApA) (A) in the presence of 1:1 (B), 2:1 (C), 3:1 (D), and 5:1 (E) molar ratios of nucleotide to gene 5 protein. Conditions were 1.0×10^{-3} M gene 5 protein, 1.0×10^{-3} M EDTA, and 2.5×10^{-2} M Tris-HCl, pH 7.5, 25 °C. Note the decrease in vertical scale expansion in (D) and (E).

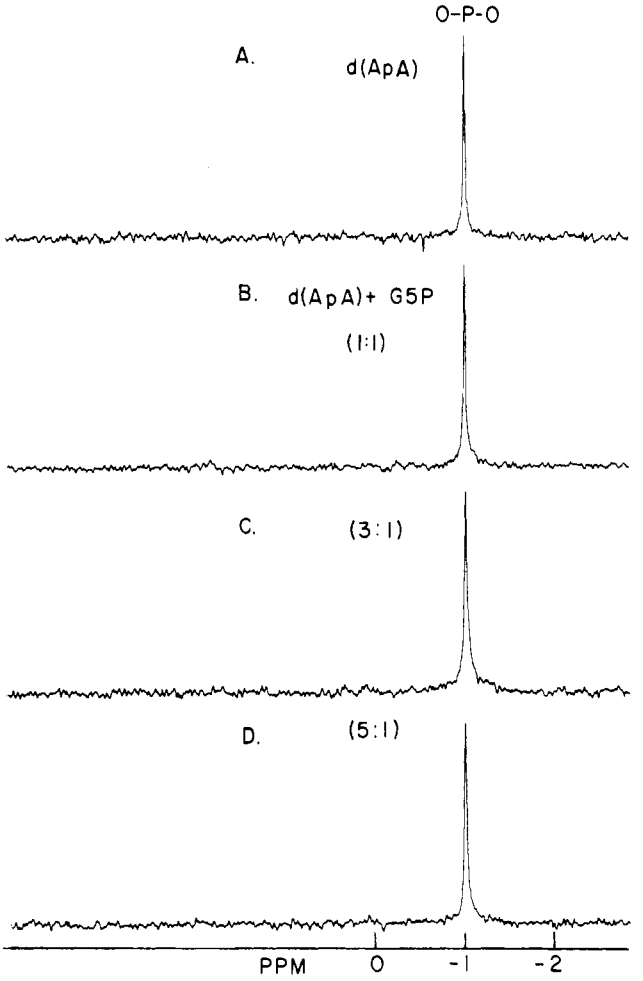

FIGURE 5: ^{31}P NMR spectra of d(ApA) (A) in the presence of 1:1 (B), 3:1 (C), and 5:1 (D) molar ratios of nucleotide to gene 5 protein. Conditions are the same as in Figure 2.

from the resonance of the diester phosphorus in the unliganded nucleotide which occurs at −0.98 ppm (Figure 4B). This suggests that all of the dinucleotide is bound despite the fact that the 5′-phosphate of a fraction of the nucleotide gives rise to a resonance near that expected for the unbound nucleotide. The minor most upfield diester resonance at −1.15 ppm appears to represent a second mode of binding of d(pApA) to gene 5 protein. The second bound form appears to be in fast exchange with free nucleotide on the NMR time scale, since addition of excess nucleotide causes the most upfield resonance to grow and shift progressively downfield (as expected for the fast exchange condition). In contrast, the downfield diester resonance of bound nucleotide remains constant in amplitude and chemical shift. Calculation of the exchange rate from the progressive downfield shift of the diester signal requires an exchange rate of no less than 90 s^{-1} for the minor bound species.

With the observation of two modes of binding for d(pApA), deduced from the diester resonance, it is possible that two modes of binding also exist for d(pA), the second mode accounting for the 20% proportion of 5′-phosphate resonance appearing near the position expected for free nucleotide in Figure 2B. Such a second mode of binding of course could not be detected in the absence of the diester resonance but might be inferred, however, by noting that the slow exchange condition for a $\Delta\delta$ of 1.5 between the bound and free 5′-phosphate requires an off constant of <760 s^{-1}. This value appears too slow to leave ~20% of nucleotide unbound at a 1:1 molar ratio and millimolar concentrations of protein and nucleotide.

^{31}P NMR Studies of Nucleotides without Free Terminal Phosphates in the Presence of Gene 5 Protein. If the 5′-terminal phosphate is removed from the dinucleotide to form d(ApA), there is practically no change (0.02 ppm upfield) in the chemical shift of the diester resonance when gene 5 protein is added (Figure 5). d(ApA) is either not bound or bound very weakly in fast exchange with free nucleotide.

The 5′-terminal phosphate was removed from $d(pA)_4$ and $d(pA)_8$ by treatment with alkaline phosphatase followed by separation of $d(A)_4$ and $d(A)_8$ from free phosphate by gel filtration or DEAE chromatography. The major peak positions of $d(A)_4$ and $d(A)_8$ in the presence of gene 5 protein are unchanged from those for the free nucleotides, suggesting little specific binding of the 5′-dephosphonucleotides (Figure 6). The broadening and evident increase in chemical shift dispersion of $d(A)_8$ suggest there is probably some weak binding of the octanucleotide. The small resonance at 1.5–1.6 ppm in the $d(A)_4$ spectra represents free phosphate which was incompletely removed by gel filtration in this case but is better removed on a DEAE-cellulose column as was done for $d(A)_8$ which shows no remaining phosphate.

^{31}P and ^{19}F NMR Studies of the Interaction of 5-Fluoro-2′-deoxyuridine 5′-Phosphate with Gene 5 Protein. In order to examine binding to gene 5 protein of both the base and phosphate portions of a mononucleotide, 5-fluoro-2′-deoxyuridine 5′-phosphate (FdUMP) was used, since this allows a simultaneous probe of base binding with ^{19}F NMR and

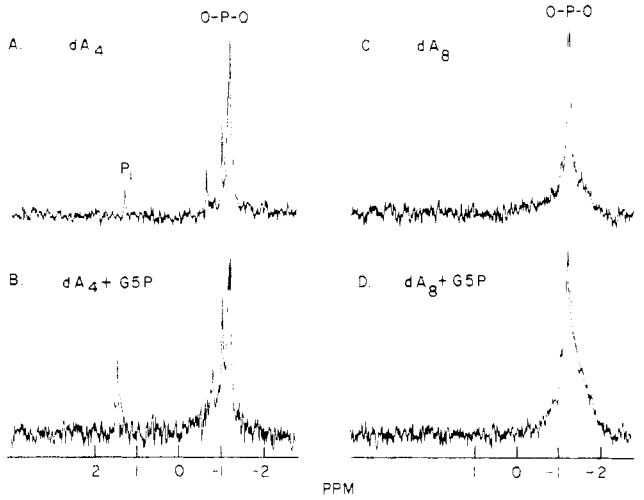

FIGURE 6: ^{31}P NMR spectra of 5′-dephosphonucleotides dA_4 and dA_8 (A and C) in the presence of gene 5 protein (B and D). Conditions were 0.76×10^{-3} M dA_4 (A and B), 0.76×10^{-3} M gene 5 protein (B), 0.56×10^{-3} M dA_8 (C and D), and 1.12×10^{-3} M gene 5 protein (D) in 2.5×10^{-2} M Tris-HCl and 1.0×10^{-3} M EDTA, pH 7.5, 25 °C.

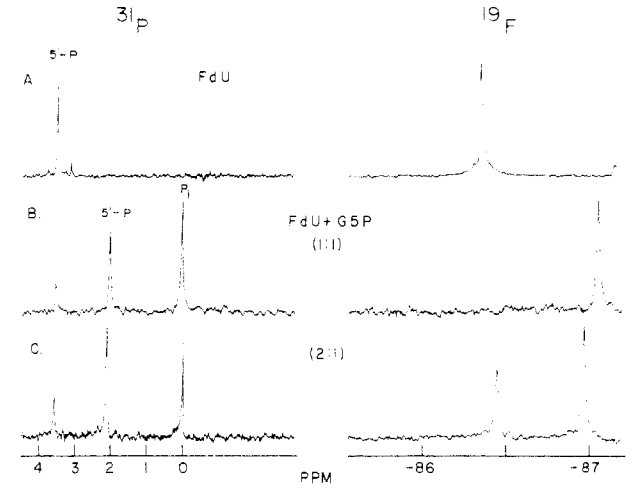

FIGURE 7: ^{31}P and ^{19}F NMR spectra of 5-fluoro-2′-deoxyuridine 5′-phosphate (FdU) bound to gene 5 protein. (Left) ^{31}P spectra: (A) free nucleotide; (B) nucleotide to gene 5 protein ratio of 1:1; (C) nucleotide to gene 5 protein ratio of 2:1. Conditions were 1.17×10^{-3} M gene 5 protein, 1.0×10^{-3} M EDTA, and 0.025 M Tris-HCl, pH 7.5, 30 °C. The "P_i" standard is an external capillary of H_3PO_4. (Right) ^{19}F spectra on the same samples as in parts A to C. Fluorine spectra are proton decoupled. In part C, the 5′ ^{31}P resonance of free nucleotide does not grow as expected because accompanying decay of the fluorouracil moiety (see text), the resonance of the sugar phosphate fragment shifts out of this region of the spectrum as well.

phosphate binding with ^{31}P NMR. The ^{31}P and ^{19}F NMR spectra obtained at gene 5 protein:FdUMP ratios of 1:1 and 1:2 are compared to the spectra of free nucleotide in Figure 7. The ^{31}P resonance of the 5′-phosphate of fluorouracil at pH 7.5 is at 3.50 ppm, similar to the 5′-phosphate of d(pA) at this pH. A fraction (~80%) of the 5′-phosphate of FdUMP shifts upfield by 1.45 ppm on binding to gene 5 protein, similar to the shift (1.6 ppm) observed on the binding of d(pA). Likewise, two 5′-phosphate resonances are present at a 1:1 molar ratio of protein and FdUMP. In contrast, the ^{19}F signal from the same sample is all shifted upfield by ~0.6 ppm from that for free nucleotide (−86.4 ppm). Hence, all the FdUMP is bound despite the presence of two ^{31}P resonances. Thus, the downfield ^{31}P resonance must represent a second form of bound nucleotide. The ^{19}F shifts are very sensitive to environment, and as the nucleotide to protein ratio is raised, there

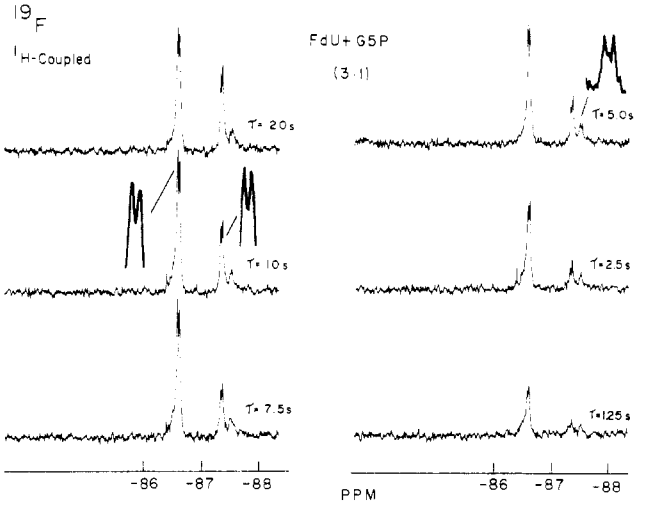

FIGURE 8: Proton-coupled ^{19}F spectra of 5-fluoro-2′-deoxyuridine 5′-phosphate (FdU) bound to gene 5 protein. Nucleotide to protein ratio is 3:1. A series of spectra with pulse delay times, τ, from 1.25 to 20.0 s is shown. Conditions were 0.70×10^{-3} M gene 5 protein, 1.0×10^{-3} M EDTA, and 0.025 M Tris-HCl, pH 7.5, 25 °C. The moderately different chemical shifts from those in the previous figure reflect the lower temperature with the decoupler off.

is a slight downfield shift of the resonance of the bound form. The initial upfield shift is compatible with a ring current shift due to the proximity to aromatic residues of the protein at the base binding site (Coleman & Armitage, 1978).

The relative resonance amplitude for the bound and free nucleotides in the presence of excess free nucleotide does not always reflect their relative concentrations, since we have discovered that in alkaline solution the free nucleotide slowly breaks down with loss of the ^{19}F resonance at this position. This process is relatively slow, but it can be significant over the time span of NMR data collection. From the chemical shift difference between the ^{19}F resonances of free and bound nucleotide, exchange between the two species must occur at less than 1064 s^{-1}.

The current model of the gene 5 protein is that of a dimer of approximately spherical monomers with a radius of ~14 Å with an isotropic tumbling time, τ_c, of $\sim 8.4 \times 10^{-9}$ s. With the assumption that all of the fluorine relaxation is due to H–F dipolar interactions, immobilized residues on a ligand bound to the protein would be expected to show a theoretical nuclear Overhauser effect ($\eta + 1$) of 0.2. The $^{19}F\{H\}$ NOE of free FdUMP is 1.06, reflecting the short τ_c. In the presence of gene 5 protein (excess nucleotide), the resonance of the unbound fraction has an NOE of 0.43, apparently reflecting viscosity and chemical exchange. The ^{19}F resonance of the protein-bound nucleotide has an NOE of 0.37. This is an averaged NOE value and will vary with the relative ratio of bound and free nucleotide. The reduction in the value of $\eta + 1$ indicates that the τ_c of the bound mononucleotide is approaching that of the protein.

T_1 values for the ^{19}F nucleus were determined by the progressive saturation method. Spectra of the complex in the presence of excess nucleotide at delay times from 1.25 to 20 s are shown in Figure 8. A second ^{19}F resonance (−87.53 ppm) corresponding to a minor form of bound nucleotide is now observed which was obscured in the proton-decoupled spectra because of a large negative η. The minor resonance is 0.16 ppm upfield of the resonance of the major bound species (−87.36 ppm) and may correspond to the minor binding mode generating the downfield ^{31}P signal. The ^{19}F nucleus on FdUMP has a T_1 of 2.3 s, while the ^{19}F nucleus of the major bound species has a T_1 of 6.6 s. This significant lengthening

of the T_1 may reflect binding of the base in a hydrophobic pocket. ^{19}F on small molecules dissolved in H_2O has relatively short T_1 values due to a relatively large dipolar contribution to the relaxation from the solvent protons (Gerig et al., 1979). Exclusion of solvent in a hydrophobic binding pocket would thus lengthen the T_1 as long as ^{1}H on amino acid side chains did not closely approach the fluorine. The ^{19}F nucleus on the minor bound species has a T_1 of 1.9 s, more like that of free nucleotide.

The coupled ^{19}F spectra of FdUMP are doublets due to the splitting of the ^{19}F resonance by the vicinal 6 proton of the uracil ring (J_{H_6F} = 6.4 Hz for both bound and free nucleotides). The magnitude of this coupling constant has been shown to be a sensitive indicator of the dihedral angle between the C_6–H_6 and C_5–F bonds in 5-fluoro-5,6-dihydrouracils and is expected to vary between 0 and ~38 Hz, depending on the dihedral angle (Byrd et al., 1978). The lack of change of this coupling constant on binding suggests that there is no distortion or other alteration in the aromatic ring on binding. The single SH of gene 5 is near one of the base binding pockets, since it can be cross-linked to T-containing nucleotides under some conditions (Paradiso et al., 1979), but no direct interaction with FdUMP appears to occur, unlike the case of thymidylate synthetase (Byrd et al., 1978).

Discussion

In vitro titrations of fd DNA or single-stranded oligonucleotides with gene 5 protein have established that each gene 5 monomer covers approximately four bases in each case (Day, 1973; Anderson et al., 1975; Coleman et al., 1976). The crystal structure of the uncomplexed protein shows it to exist as a dimer of tightly interlocking monomers (McPherson et al., 1979), a structure that probably exists in solution. The DNA binding faces of the two constituent monomers of the dimer appear to be separated on the exterior surfaces of this dimeric structure (McPherson et al., 1980a,b). These findings and solution NMR work have led to a model in which the fd DNA is wound on the outside of a core structure of bound gene 5 protein dimers (Coleman & Armitage, 1978). This would account for the collapse of the circular DNA into a long cigar-shaped structure as observed for both in vivo and in vitro complexes of gene 5 protein with circular single-stranded fd DNA (Pratt et al., 1974). At any one cross-section of the complex, the two opposite regions of the DNA strand brought to close proximity by binding to the gene 5 dimer would be bound in an antiparallel fashion, since the binding faces are oriented in opposite directions across the 2-fold axis of the dimer. The tight interaction between the monomers of the dimer, perpendicular to the long axis of the complex with a polynucleotide, probably requires that in a $d(pA)_4$ complex the minimal structure is two $d(pA)_4$ molecules bound to opposite faces of the dimer. In the $d(pA)_8$ complex, the minimal homogeneous complex must be a tetramer, two dimers bound together side to side by two oligonucleotides occupying the two in-line binding surfaces of adjacent dimers.

Specific Interaction of the 5′-Phosphate of Oligonucleotides with Gene 5 Protein. Studies of gene 5 protein–nucleotide interactions have assumed that oligonucleotide binding to gene 5 protein approximates polynucleotide binding, although the 5′-terminal phosphate dianion on the former is a potential binding locus not present "in vivo". Titration studies show the same stoichiometry of base interaction for oligo- and polynucleotides (Coleman et al., 1976), although the high-resolution NMR methods which have supplied the most structural details unfortunately cannot be applied to the polynucleotide complexes with long rotational correlation times. Methods applicable to both oligo- and polynucleotide complexes have not suggested major differences in binding of oligo- and polynucleotides. The ^{31}P NMR methods, while not useful for detailed structural interpretation, do reveal that the 5′-phosphate dianion of the oligonucleotides is involved in a highly specific interaction with the protein which influences phosphodiester conformation as well (Figures 1 and 6, 4 and 5). Whether the same rigid conformation present in the $d(pA)_4$ and $d(pA)_8$ complexes is achieved in the presence of an infinite string of singly charged diester phosphates in a polynucleotide complex must remain speculative. Such a rigid conformation is clearly not achieved by a short stretch of diester phosphates (Figure 6).

Complex formation with the octanucleotide would be expected to introduce additional cooperative side to side interactions of the protein dimers, more characteristic of the polynucleotide complex. This additional interaction appears to be reflected in the decrease in the dissociation constant from $\sim 10^{-6}$ M for $d(pA)_4$ to $<10^{-9}$ M for $d(pA)_8$ (Coleman et al., 1976). The latter magnitude is similar to that estimated for the dissociation constant for the complex with fd DNA. The broad line in the spectrum of the $d(pA)_8$ complex (Figure 1) most probably represents formation of a few larger aggregates of gene 5 protein held together by octanucleotides which bind to more than two monomer faces, as was previously suspected from the observation of considerable broadening in the ^{1}H NMR spectrum of the octanucleotide complexes observed on initial formation of the complex (Coleman et al., 1976).

The small upfield chemical shifts on binding of oligonucleotides suggest some change from the gauche–trans configuration of the diester expected of a fully extended nucleotide. The upfield shifts (<0.2 ppm) are not of the magnitude (1–2 ppm) expected for complete transition to the gauche–gauche conformation, found for example in B-DNA. Hence, the complexed oligonucleotide probably remains in a fairly extended form. The same three phosphodiester conformations are preserved in the octanucleotide complex (Figure 1C), even though one phosphodiester bond must span the interface between two gene 5 monomers. The side to side interaction of the monomers in the stacked gene 5 dimers must maintain a completely regular winding of the phosphodiester backbone, in fact the same as within one monomer.

Binding of Mono- and Dinucleotides to Gene 5 Protein. Binding studies with nucleotides that do not fill the binding locus on gene 5 monomer, i.e., less than four nucleotide residues in length, have not been carried out with the gene 5 system. Extensive studies using nucleotides (from one to eight residues in length) that do not fill the single-stranded DNA binding locus of gene 32 protein from T4 have been published by Kowalczykowski et al. (1981). One might assume that with four potential base-binding pockets available in the gene 5 binding lattice, a mononucleotide would bind loosely and relatively equally to each locus with saturation of binding requiring ratios of nucleotide to protein approaching 4:1. Instead the ^{31}P data show that mono- and dinucleotides prefer a single binding mode, which is largely established at a 1:1 molar ratio of nucleotide to protein and is characterized by an upfield shift of the ^{31}P resonance of the terminal phosphate dianion by ~1 ppm (Figures 1–4 and 7). This must reflect a specific interaction of the dianion with one unique site on each monomer.

Chemical modification data show that the lysyl residues of the protein also participate in binding of DNA to gene 5 protein (Anderson et al., 1975). The electrostatic interaction of the ϵ-NH_3^+ groups with the phosphate backbone may supply

a large component of the stabilization energy, although the ^1H NMR suggests that specific stable hydrogen bonds may not form with the diester backbone (Coleman et al., 1976). The upfield shift of the resonance of the 5′-phosphate dianion would be adequately explained by a salt bridge(s) between specific lysyl residues and the dianion. Such a specific interaction could be described as limiting the protein interaction with the oligonucleotide lattice regardless of the length of the oligonucleotide, since this major binding interaction would be the same for $d(pN)_1$ to $d(pN)_8$, despite the fact that all of the DNA binding surface of the protein is not covered by nucleotides less than four residues in length. Thus, a limited "lattice" interaction, similar to that observed by Kowalczykowski et al. (1981) for gene 32 protein, would relate in this case to specific placement of the phosphate dianion rather than to conformational barriers to interaction at other base-binding positions on the protein.

Both the ^{31}P and ^{19}F NMR spectra detect a minor binding mode (Figures 4 and 7), which may represent binding to other available "base-binding pockets" which place the 5′-phosphate interaction out of register and relatively free. The latter may account for the lack of shift of the ^{31}P resonance of the 5′-phosphate in this mode.

The prominent role of the 5′-phosphate dianion in contributing to the binding energy of the model oligonucleotides (mono to octa) to gene 5 protein, probably through electrostatic forces, suggests that certain features of their complexation with gene 5 protein may be significantly different from those present in an essentially infinite string of bound diesters in the true DNA complexes. In the case of polynucleotides or DNA complexes with gene 5 protein, the cooperative protein–protein interactions between adjacent monomers are probably much more prominent in determining the overall stability of the complex.

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