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Structure of Gene 5 Protein–Oligodeoxynucleotide Complexes as Determined by ^{1}H, ^{19}F, and ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: ^{1}H nuclear magnetic resonance (NMR) spectra at 270 MHz of gene 5 protein from bacteriophage fd and its complexes with tetra- and octadeoxynucleotides show that ~12 of the 37 aromatic protons of the protein undergo upfield shifts upon nucleotide binding. In the complex with $d(pT)_8$, the upfield shifts of the aromatic protons average ~0.3 ppm, while in the $d(pA)_8$ complex the same resonances (assigned to tyrosyl protons) shift upfield ~0.8 ppm. These are interpreted as ring current shifts induced by stacking of the phenyl rings of three of the five tyrosyl residues with the bases of the nucleotides. ^{19}F NMR of *m*-fluorotyrosyl gene 5 protein shows five separate resonances: two downfield from *m*-fluorotyrosine corresponding to "buried" tyrosyls and three near *m*-fluorotyrosine corresponding to "surface" tyrosyls. The latter (assigned to Tyr-26, -41, and -56, shown by chemical modification to be exposed to solvent) move upfield on nucleotide binding. The downfield ^{19}F resonances are unaffected. Thus the aromatic protons shifted upfield on nucleotide binding appear to be those of Tyr-26, -41, and -56. In contrast to tetra-, octanucleotide binding to gene 5 protein induces large changes in the ^{1}H resonances of the $-CH_3$ groups of the Val, Leu, and Ile side chains. These may reflect conformational changes induced by protein–protein interactions between two monomers bound to the octanucleotide. ^{1}H resonances of the ϵ-CH_2 groups of the lysyl residues in the protein and the complexes with nucleotides are narrow with long T_2 values, suggesting considerable rotational motion. Thus ϵ-NH_3^+–phosphate interactions, if they occur, are on the surface of the complex and allow the ϵ-CH_2 groups to retain considerable rotational freedom. ^{31}P NMR of the bound nucleotides shows large decreases in T_1 for the 3′–5′ diesters, but little chemical shift suggesting no unusual distortion of the nucleotide backbone on binding to gene 5 protein. A three-dimensional model of a gene 5 protein–octanucleotide complex has been built based on predictions of the secondary structure from the amino acid sequence (87 AA) and tertiary folding dictated by known chemical and NMR features of the complex.

Gene 5 protein, the product of gene 5 of the filamentous bacteriophages fd, fl, or M13, is a small (mol wt 9689) DNA-binding protein specific for single-stranded DNA (Alberts and Frey, 1970; Alberts et al., 1972; Oey and Knippers, 1972). Its major physiological function appears to be the formation of a stoichiometric complex with the single-stranded DNA of the daughter virions and thereby prevent their further use as templates for the synthesis of double-stranded replicative forms, RF[1] (Salstrom and Pratt, 1971; Mazur and Model, 1973; Pratt et al., 1974). There is some evidence that gene 5 protein may also play a positive role in initiating the synthesis of the single-stranded daughter genomes from the double-stranded RF's (Staudenbauer and Hofschneider, 1973). Each gene 5 protein covers approximately four nucleotides as determined by titration studies using the changes in optical density (Alberts et al., 1972) or circular dichroism (Day, 1973; Anderson et al., 1975) of the base chromophores on the binding of gene 5 protein to fd DNA. The protein will bind to deoxynucleotides containing any of the four common bases in any sequence, although there are marked variations in binding affinity (see below). The physiochemical characteristics of the complex between gene 5 protein and DNA have been investigated extensively (Alberts and Frey, 1970; Oey and Knippers, 1972; Alberts et al., 1972; Day, 1973; Anderson et al., 1975; Pretorius et al., 1975; Cavalieri et al., 1976) and will not be reviewed in detail here.

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; CD, circular dichroism; RF, replicative form; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Binding of gene 5 protein to single-stranded DNA is cooperative in that a free gene 5 monomer prefers to bind adjacent to a protein monomer already bound to the DNA than to an isolated site on the DNA (Alberts et. al., 1972; Cavalieri et al., 1976). Gene 5 protein will form a complex with an isolated tetranucleotide with a dissociation constant of $\sim 10^{-6}$ M, while the complex with fd DNA has a dissociation constant of $\sim 10^{-9}$ M (Anderson et al., 1975). The additional "cooperative" binding affinity is presumably contributed by protein–protein interactions between adjacent monomers in the complex. The small size of this protein, its known amino acid sequence (Nakashima et al., 1974b; Nakashima and Konigsberg, 1975), and the ability to form complexes with small defined oligodeoxynucleotides makes this an ideal system in which to apply NMR methods for the determination of molecular structure in solution. The present paper presents initial studies using ^{1}H, ^{19}F, and ^{31}P NMR to explore the solution structure of this protein and its deoxynucleotide complexes.

Experimental Section

Gene 5 Protein. Homogeneous gene 5 protein was prepared by DNA-cellulose chromatography as previously described (Alberts and Herrick, 1971; Anderson et al., 1975). The preparation of *m*-fluorotyrosyl gene 5 protein was carried out as described in Anderson et al. (1975). For the ^{1}H NMR studies concentrated gene 5 protein was exchanged against 99.8% D_2O containing 10 mM DPO_4^{2-} at pH 8.0[2] by five 6-h dialyses of 1 ml of protein against 10-ml volumes of the D_2O buffer. Deuterated phosphate was prepared by repeated lyophilization from 99.8% D_2O.

Nucleotides. Tetra- and octanucleotides of defined sequences were purchased from Collaborative Research (Waltham, Mass.). Tetranucleotides of random sequence were prepared from a limit digest by pancreatic DNase of calf thymus DNA using column chromatography as described by Tomlinson and Tener (1963). For the NMR studies the lyophilized nucleotides were dissolved in the required volume of 99.8% D_2O–10 mM DPO_4^{2-}, pH 8.0. Nucleotides were added to the protein solutions by lyophilizing the correct volume and concentration of nucleotide from D_2O buffer and adding the lyophilized nucleotide to the protein solution.

Absorption and CD Spectra. Absorption spectra were measured on a Cary 15 spectrophotometer. For calculations of the concentration of gene 5 protein, an $E_{276}^{0.1\%} = 0.73$ was employed (Day, 1973). Circular dichroism was measured with a Cary 61 spectropolarimeter.

NMR Methods. ^{1}H NMR spectra were recorded on a FT 270-MHz Bruker spectrometer with an Oxford Instrument Co. superconducting magnet coupled to a Nicolet 1085 computer with a 293 pulse controller and a 294 disc system. D_2O present in the sample served as a field-frequency lock. Pulse sequences for the simplification of protein NMR spectra were applied as described in Campbell et al. (1975). Irradiation of the proton resonance of water was carried out by the gated decoupling technique (Hoult and Richards, 1975). Measurements were made at 25 °C, and chemical-shift values are reported as parts per million (ppm) downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

^{31}P NMR spectra were recorded on a FT-Bruker HFX-90 spectrometer operating at 36.4 MHz as previously described (Chlebowski et al., 1976). Measurements were made at 25 °C and chemical shifts in ppm are reported relative to 85% H_3PO_4. Spin–lattice relaxation times (T_1) were determined by progressive saturation and/or the inversion–recovery method (Vold et al., 1968).

^{19}F NMR spectra were recorded on a FT-Bruker HFX-90 spectrometer operating at 84.6 MHz. D_2O present in the sample served as the field-frequency lock and chemical shifts are reported in ppm relative to CF_3COOH. Samples were contained in 0.01 M Tris-HCl, 90% H_2O–10% D_2O, pH 8.0, 25 °C. Initial studies on the fluorotyrosyl gene 5 protein system were carried out on a Varian XL-100-15 spectrometer for which we thank Drs. Brian D. Sykes and William E. Hull.

[2] pH will refer throughout to the actual reading (on either D_2O or H_2O solutions) taken on a Radiometer pH meter, Model 26, fitted with a GK2321C electrode.

Results

The 270-MHz ^{1}H NMR, Gene 5 Protein–Octanucleotide [$d(pT)_8$] Complex. The proton spectrum of native gene 5 protein (dialyzed against 99.8% D_2O–0.01 M DPO_4^{2-}) at 270 MHz is shown in Figure 1A. The C(2)H resonance of the single histidyl, His-64, is present at 8.02 ppm (peak 1). A number of overlapping aromatic resonances containing the -CH resonances from the five Tyr and three Phe residues and the C(4)H of His-64 appear in the region 6.5–7.5 ppm (peaks 3 to 9). The integrated area under these resonances approximates the 36 protons expected using the single C(2)H histidine proton as the standard.

In the high-field region of the spectrum, the ϵ-CH_2 groups of the six Lys residues are resolved in a narrow line at 3.1 ppm. The δ-CH_2 resonances of the four Arg residues occur at 3.2–3.5 ppm. A large group of overlapping resonances from the methyl groups of the aliphatic residues occurs from 0.5 to 2 ppm and these are assigned as indicated in Figure 1A. There are a few high-field resonances near 0 ppm (peaks a, b, and c) which represent methyl resonances shifted upfield by ring currents. These assignments are made on the basis of the previous ^{1}H NMR work on proteins (Roberts and Jardetzky, 1970; Dwek, 1976; Campbell et al., 1974, 1975; Dobson et al., 1975; Bradbury et al., 1973).

The 270-MHz ^{1}H spectrum of the same sample of gene 5 protein after the addition of 1 mol of $d(pT)_8$ per 2 mol of protein is shown in Figure 1B. Significant changes occur in both the line width and the chemical shift of a number of the resonances of the aromatic and aliphatic protons of the protein on formation of the octanucleotide complex. These changes are best visualized in the difference spectrum A − B (Figure 1). In A − B the resonances above the baseline are those that have disappeared from the protein on complex formation, while the inverted crosslined peaks are new resonances appearing in the complex. Because of slight aggregation induced by the octanucleotide, it is difficult to match precisely the concentrations of the protein and the octanucleotide complex. Thus the difference spectrum in the aromatic region has been computed by assuming that the resonance of the C(2)H proton of His-64 is unchanged in the complex (compare Figures 1A and 1B). In the aliphatic region of the spectrum the difference has been computed by assuming that the magnitude of the resonance from the lysyl ϵ-CH_2 groups is unchanged on complex formation (see Figures 1 and 3). The spectrum of free $d(pT)_8$ under the same conditions is shown in Figure 1C.

While individual resonances of many of the aromatic protons overlap, major peaks occurring in the spectra of the protein, the complex with $d(pT)_8$, and the difference spectrum are well resolved and have been numbered 1 to 10 in Figure 1. Their chemical shifts and proposed assignments are given in Table I. On formation of the octanucleotide complex, peaks 3, 4, and 9 disappear from the aromatic region of the protein spectrum,

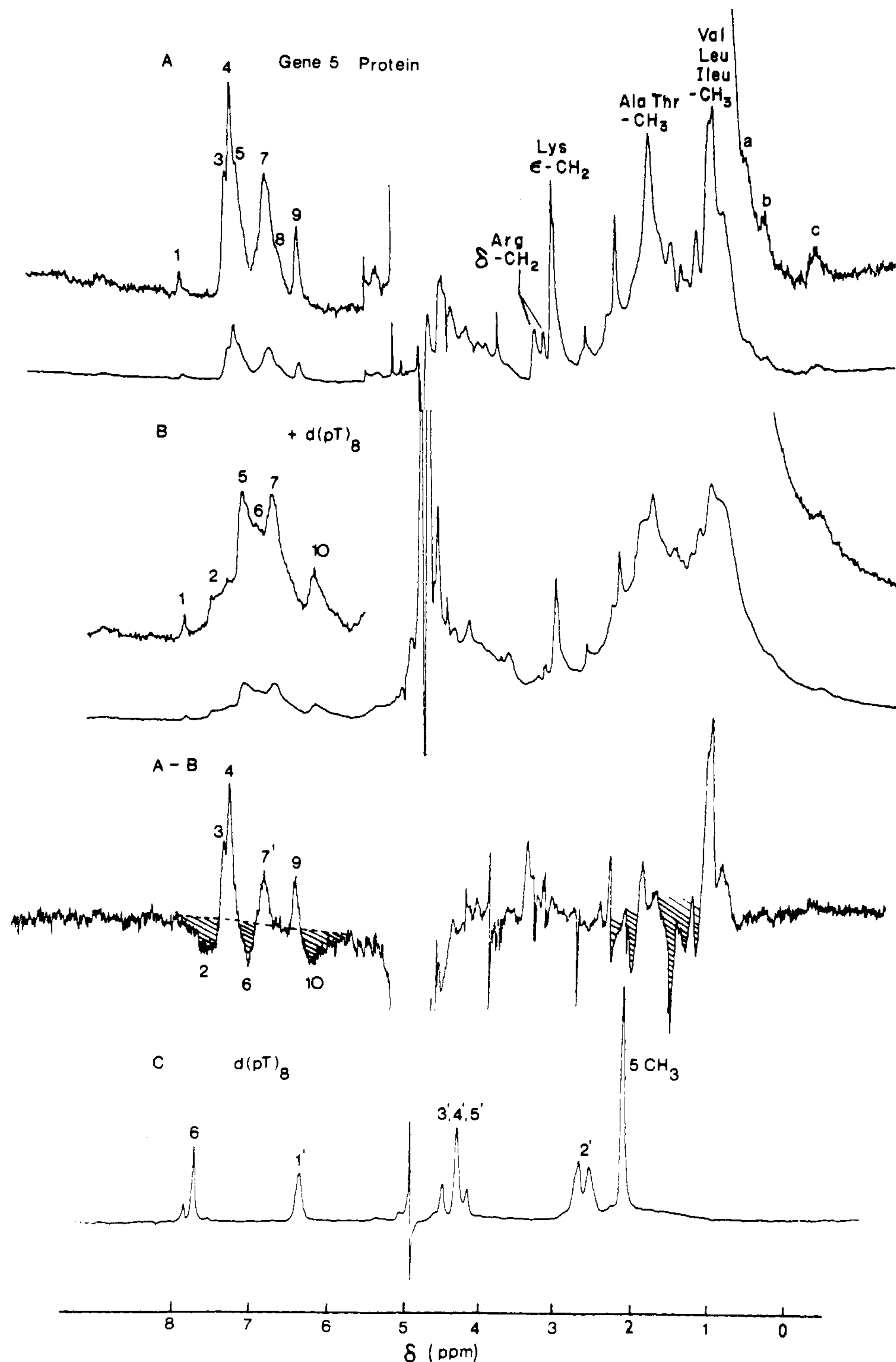

FIGURE 1: The 270-MHz ^{1}H NMR of gene 5 protein, 2×10^{-3} M (A); plus 1×10^{-3} M $d(pT)_8$ (B). A − B is the difference spectrum plotted by setting the C(2)H histidyl resonance (peak 1) and the lysyl ε-CH_2 resonance at 3.1 ppm at the same amplitudes in both spectra. (C) The 270-MHz ^{1}H NMR of $d(pT)_8$, 3×10^{-3} M. Conditions: 0.01 M DPO_4^{2-}, pH 8.0, 25 °C. On the nucleotide spectra, primed and unprimed numbers will refer to the carbon atom carrying nonexchangeable protons in the sugar and base, respectively.

while peak 1 (the C(2)H of His-64) remains unchanged as do peaks 5, 8, and most of peak 7. In spectra of the protein at lower concentration, peak 8 is clearly resolved and corresponds to one proton and has thus been assigned to the C(4)H of His-64 (see Figure 5 below). A part of peak 7, 7′, disappears in the spectrum of the complex. Peak 2 in the complex, which contains at least two resonances of different chemical shift, appears to represent a broadened resonance arising from the C(6)H protons of the thymine ring. Since the lines are broadened in the spectrum of the $d(pT)_8$ complex, it is difficult to match exactly resonances disappearing from the spectrum of the free protein with new resonances appearing in the complex. Qualitatively, however, resonance from peaks 3 and 4 in the spectrum of the protein would appear to be present near 7 ppm (peak 6) in the spectrum of the complex, while resonance of peaks 7 and 9 appears in the broad region labeled peak 10 in the complex. It is also possible that resonances from some of the protons in the complex are broadened beyond detection.

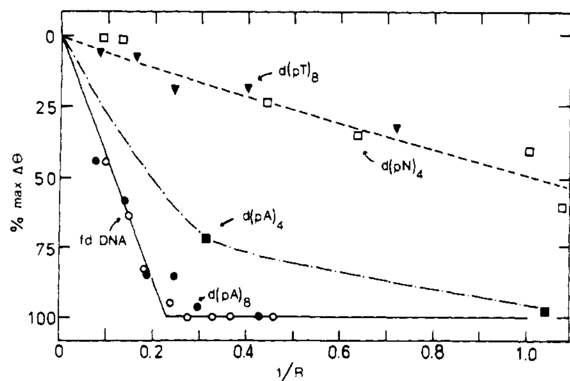

FIGURE 2: Change in nucleotide ellipticity, % maximum $\Delta\theta$, at 260 and 270 nm as a function of the reciprocal of the base: protein ratio, R. (○) fd DNA; (●) d(pA)$_8$; (■) d(pA)$_4$; (▼) d(pT)$_8$; (□) d(pN)$_4$. Conditions: (1-3) $\times$ 10^{-5} M nucleotide or DNA; 0.01 M Tris-HCl, pH 8.0, 25 °C.

The sum of the areas under peaks 3, 4, and 7′ of A − B (Figure 1) represents ~35% of the original area under the aromatic resonances of the protein (Figure 1A). Thus the environments of about one-third of the aromatic protons of the protein are altered on nucleotide binding, compatible with the assignment of these particular resonances to the aromatic protons of the three tyrosyl residues (Tyr-26, -41, and -56) that are known on other grounds to interact with the nucleotide (see Discussion). These resonances appear to undergo upfield shifts of from 0.17 to 0.6 ppm on nucleotide binding (Table I).

The proton resonances of the methyl groups of the branched aliphatic side chains of the protein undergo broadening as well as significant chemical shifts on octanucleotide binding as represented by a number of positive and negative peaks in the high-field region of the difference spectrum (A − B, Figure 1). Which of the negative peaks represents the protons of the thymidine methyl groups in the complex is not certain, but they might include one or both peaks at 2.0 and 1.5 ppm. The resonances of the ϵ-CH_2 protons of the lysyl residues at ~3.1 ppm undergo neither broadening nor significant chemical shift in the complex and will be examined in more detail below. One of the resonances (that at lower field) attributed to the δ-CH_2 protons of the arginyl residues disappears in the spectrum of the d(pT)$_8$ complex.

Nearly identical changes are seen in the aromatic region of the 270-MHz ^{1}H NMR spectrum of gene 5 protein when tetranucleotides of random sequence are added at a 1:1 molar ratio of nucleotide to protein (not shown). In contrast to the octanucleotide complex, only minor changes occur in the envelope of the aliphatic resonances of the tetranucleotide complex.

Relative Binding Affinity of Tetra-, Octa-, and Polydeoxynucleotides for Gene 5 Protein. Gene 5 protein appears to bind tightly to single-stranded DNA regardless of the base sequence (Alberts et al., 1972; Anderson et al., 1975; Cavalieri et al., 1976). However, in examining the binding of gene 5 protein to the oligodeoxynucleotides of defined sequence used in the NMR studies, we have discovered that gene 5 protein has substantially higher affinity for adenine-containing nucleotides than for thymine-containing nucleotides. The differential binding of gene 5 protein to tetranucleotides, octanucleotides, and fd DNA is shown by the data in Figure 2 which are derived from the binding assay using the change in the CD of the nucleotides at 260 to 270 nm induced by the binding of gene 5 protein as previously described (Anderson et al., 1975).

TABLE I: Chemical Shifts and Proposed Assignments of Aromatic Proton Resonances in Gene 5 Protein and Its Complex with d(pT)$_8$.

Peak No.	Chemical Shift (ppm)	Proposed Assignment
1	8.02	C(2)H His-64 (protein and complex)
2	7.65–7.45	C(6)H Thymine (complex)
3	7.42	Tyr (protein)
4	7.35	Tyr (protein)
5	7.28	Phe (protein and complex)
6	7.05	Tyr (complex)
7	6.90	Tyr and Phe (protein and complex)
7′	6.90	Tyr (protein)
8	6.72	C(4)H His-64
9	6.49	Tyr (protein)
10	6.33	Tyr (complex)
	Ring Current Shifts in Complex (ppm)	
3	0.37	Tyr 26, 41, and 56
4	0.30	Tyr 26, 41, and 56
9	0.17	Tyr 26, 41, and 56
7′	~0.6	Tyr 26, 41, and 56

The percent maximum change in the CD is plotted as a function of the reciprocal of the base:protein ratio, R.

For the binding of gene 5 protein to fd DNA, the CD titration is sharp with an endpoint near a $1/R$ value of 0.25 or an R value of 4 bases per protein monomer. Binding of gene 5 protein to d(pA)$_8$ results in a similar titration, also complete at a $1/R$ value of 0.25 (Figure 2). In contrast, the tetranucleotides of random sequence bind much less tightly. At the ~10^{-5} M concentrations employed in these titrations, a large excess of protein is required to saturate the nucleotide (Figure 2). The dissociation constant, K_d, calculated from these data for the complex of gene 5 protein with tetranucleotides of random sequences is ~3×10^{-6} M. In marked contrast to d(pA)$_8$, d(pT)$_8$ binds no more tightly to gene 5 than do the tetranucleotides of random sequence, $K_d = 3 \times 10^{-6}$ M. On the other hand, d(pA)$_4$ (K_d = ~10^{-7} M) binds more tightly than d(pT)$_8$. From the data in Figure 2, the maximum K_d values for d(pA)$_8$ and fd DNA are 5×10^{-8} M and they may be considerably smaller. All of these K_d values ensure that, at the mM concentrations of protein and nucleotide employed in the NMR experiments, saturation of the protein binding site is achieved near the ratio of 1 protein monomer to 4 bases.

The 270-MHz ^{1}H NMR, Gene 5 Protein–Octanucleotide [d(pA)$_8$] Complex. In view of the much larger affinity of adenine-containing nucleotides for gene 5 protein compared with thymine-containing nucleotides, the effects of d(pA)$_8$ on the ^{1}H NMR spectrum of gene 5 protein were examined. The proton NMR spectrum of this complex is more complicated because of the multiple resonances of the nucleotide spectrum arising not only from the presence of the C(8)H and C(2)H aromatic protons of adenine, but also from the splitting of these resonances as well as those of the sugar by the base stacking that occurs in adenine-containing nucleotides (Figure 3C). The spectrum of the sample of gene 5 protein used to form the d(pA)$_8$ complex is shown in Figure 3A and that of the complex formed by adding a 1:2 molar ratio of d(pA)$_8$ to protein is shown in Figure 3B. In the upfield aliphatic proton region of

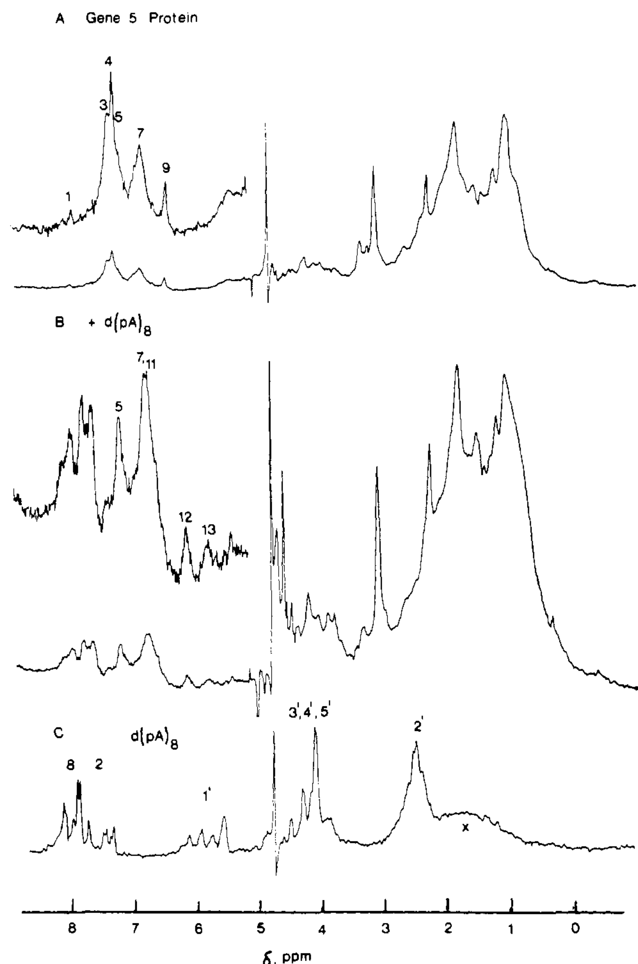

FIGURE 3: The 270-MHz ^{1}H NMR of gene 5 protein, 1.5×10^{-3} M (A); plus 0.75×10^{-3} M $d(pA)_8$ (B); (C) 270-MHz ^{1}H NMR of $d(pA)_8$, 1.5 $\times$ 10^{-3} M. Conditions: 0.01 M $DPO_4{}^{2-}$, pH 8.0, 25 °C. Peak X is a baseline artifact.

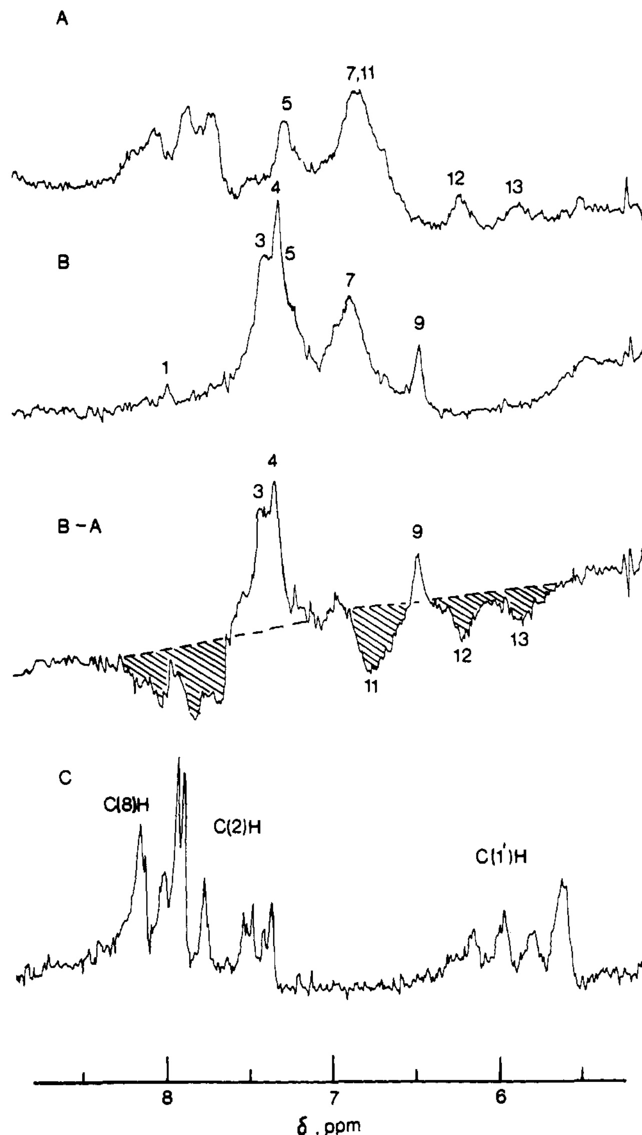

FIGURE 4: The 270-MHz ^{1}H NMR of the aromatic protons of the gene 5 protein-$d(pA)_8$ complex (A); of gene 5 protein alone (B); and the difference spectrum (B $-$ A), plotted by setting the ϵ-CH_2 resonance (not shown) at the same magnitude in both A and B; (C) 270-MHz ^{1}H NMR spectrum of $d(pA)_8$, 1.5×10^{-3} M, in the downfield region. C(8)H and C(2)H identify the general location of the resonances corresponding to these protons in adenine, but specific assignments are not indicated. Conditions as in Figure 3.

the NMR spectrum, changes occur on complex formation which are very similar to those observed when $d(pT)_8$ is added. The difference spectrum, assuming the lysyl ϵ-CH_2 resonances to be unchanged, is in fact very similar in the upfield region to that shown in Figure 1 and is not shown here. As in formation of the $d(pT)_8$ complex, some aggregation of the protein is induced by $d(pA)_8$ which makes it difficult to match spectral amplitudes of the complexes with the free protein.

The aromatic proton spectrum of the $d(pA)_8$ complex is complicated by the adenine protons, but the qualitative changes in the aromatic proton resonances assigned to the protein can easily be recognized. These changes are seen in more detail in the expanded aromatic spectra of the complex (Figure 4A), the native protein (Figure 4B), and $d(pA)_8$ (Figure 4C). As shown in the difference spectrum (B $-$ A), the downfield peaks 3 and 4 of the protein (assigned to tyrosyl protons, Table I) have disappeared from the spectrum of the complex as has peak 9 of the protein (also assigned to tyrosyl protons, Table I). These three changes are also characteristic of the formation of the tetranucleotide and $d(pT)_8$ complexes (see Figure 1). In contrast to the $d(pT)_8$ complex, no resonance appears in the position of peak 6 (7.05 ppm). The first new resonance appearing in the $d(pA)_8$ complex is a piling up of resonance on the upfield edge of peak 7 at ~6.7 ppm (peak 11, Figure 4). Thus the downfield tyrosyl protons of the protein appear to have shifted ~0.8 ppm upfield in the $d(pA)_8$ complex compared with 0.30 to 0.37 ppm in the $d(pT)_8$ complex. Likewise the tyrosyl proton resonance at 6.49 ppm in the protein appears to have shifted upfield, but its exact position is difficult to identify in the complex since peaks 12 and 13 in the complex may also contain some of the C(1′)H resonances of the sugar residues. If the tyrosyl proton resonances originally appearing at peak 9 are assigned to peak 12, they have shifted upfield by ~0.18 ppm, slightly greater than the shift observed in the $d(pT)_8$ complex.

The group of resonances from 7.5 to 8.5 ppm assignable to C(8)H and C(2)H of the adenine bases have undergone considerable rearrangement in the complex. The most intense resonances from the aromatic protons of the bases in the complex occur at 7.7 and 7.8 ppm compared with 7.9 and 8.2 ppm for the most intense resonances for this group of protons in free $d(pA)_8$ (Figure 4). The group of peaks representing the resonances of the C(1′)H of the deoxyribose residues have also

TABLE II: Chemical Shifts and Proposed Assignments of Proton Resonances in the Gene 5 Protein-d(pA)$_8$ Complex.

Peak	Chemical Shift (ppm)	Proposed Assignment
Major aromatic resonances of adenine in the free nucleotide	8.20	C(8)H of adenine
	8.08 } 8.00	C(8)H of adenine
	7.96	C(8)H and/or C(2)H of adenine
	7.83	C(8)H and/or C(2)H of adenine
	7.58 } 7.53	C(2)H of adenine
	7.46 } 7.40	C(2)H of adenine
C(1′)H resonances of the sugars in the free nucleotide	6.20	C(1′)H of deoxyribose
	6.00	C(1′)H of deoxyribose
	5.80	C(1′)H of deoxyribose
	5.66	C(1′)H of deoxyribose
Major aromatic resonances of adenine in the complex	8.21	C(8)H and C(2)H of adenine
	8.06	C(8)H and C(2)H of adenine
	7.88	C(8)H and C(2)H of adenine
	7.73 } 7.70	C(8)H and C(2)H of adenine
Protein Resonances in the gene 5 protein-d(pA)$_8$ complex		
5	7.28	Phe (unshifted in complex)
7	6.90	Tyr (unshifted in complex)
11	6.76	Tyr (ring current shifted in complex)
12	6.23	Tyr (ring current shifted in complex) and C(1′)H of deoxyribose
13	5.91	C(1′)H of deoxyribose

been rearranged in the complex and the major peaks, 12 and 13, assignable to these protons (and possibly also to the tyrosyl protons of peak 9) are of different intensity and occur in different positions than the C(1′)H resonances of the free nucleotide. This suggests that at the very least the stacking of the adenine bases has been altered in the complex. some of the aromatic protons of the adenine bases are probably also undergoing upfield shifts in the complex. The chemical shifts of the major low-field resonances of d(pA)$_8$ and its complex with gene 5 protein are listed in Table II.

General Structure of Gene 5 Protein. The 270-MHz ^{1}H NMR spectrum of native gene 5 protein at a lower concentration than presented in the earlier figures is shown in Figure 5A. The resonance assigned to the C(4)H of His-64 (peak 8) is more clearly resolved in this spectrum. Two relatively intense narrow resonances occur in the upfield region. One, at ~3.1 ppm, has been assigned to the ϵ-CH_2 groups of the lysyl residues and the line width and chemical shift of this resonance are unchanged when the protein is denatured in sodium dodecyl sulfate (Figure 5C). The second narrow resonance occurs at ~2.1 ppm and has been assigned to the methyl protons of the two methionyl residues.

The 270-MHz ^{1}H spectrum of 2 mM gene 5 protein in 0.01 M borate buffer, pH 8.0, is shown in Figure 5B. Extensive changes occur in both the aromatic and aliphatic resonances compared with the spectrum of the protein in phosphate buffer. The aromatic spectrum of the protein collapses to occupy a narrower range of chemical-shift values and peak 9 (assigned to upfield shifted tyrosyl resonances, Table I) has almost completely disappeared. The aromatic proton spectrum of the protein in borate buffer is similar to that of the protein in sodium dodecyl sulfate (Figure 5C). In borate the resonances from the α-CH protons of the peptide backbone have become more distinct and many of the methyl resonances of the branched aliphatic side chains have coalesced into a large resonance at ~1 ppm. The ring current shifted methyl resonances between 0 and 1 ppm have disappeared. All of these changes suggest a transformation of the protein structure to a random coil. No evidence of nucleotide binding to the protein in the presence of borate buffer could be obtained by NMR methods. Deleterious effects of borate buffer on nucleotide binding proteins have recently been reported (Johnson and Smith, 1976).

Pulse sequences (90°–τ–180°–τ), the Carr–Purcell method A (Campbell et al., 1975) applied to gene 5 protein with delay times, τ, progressively increased from 15 to 30 to 45 ms are shown in Figure 6. With the use of this pulse sequence, the area of the resonances decay exponentially with a time constant of T_2. Hence resonances with short T_2 values (less than τ) rapidly decay to zero and are not observed. From the series of spectra in Figure 6, most of the proton resonances in gene 5 protein have rather short T_2 values since only at τ = 15 ms do significant aromatic or branched aliphatic resonances remain. Only the protons of the ϵ-CH_2 groups of the lysyl residues and the methyl groups of the methionyl residues have relatively long T_2 values, probably in the neighborhood of 30 to 40 ms, although no exact value can be derived from the Carr–Purcell A sequence.

^{19}F NMR of Fluorotyrosyl Gene 5 Protein–Tetranucleotide Complex. Fluorotyrosyl gene 5 protein synthesized by infecting an *Escherichia coli* tyrosine auxotroph growing on *m*-fluorotyrosine with fd has the same DNA binding and functional properties as the native protein (Anderson et al., 1975). The ^{19}F NMR spectrum of the uncomplexed protein is shown in Figure 7A, while the spectra in the presence of 1 equiv and 3 equiv of tetranucleotide (random sequences) are shown in Figures 7B and 7C, respectively. In the uncomplexed protein resonances from two of the fluorotyrosyl residues appear 2.8 and 2.2 ppm downfield from the resonance position for free *m*-fluorotyrosine, while the resonances of three of the fluo-

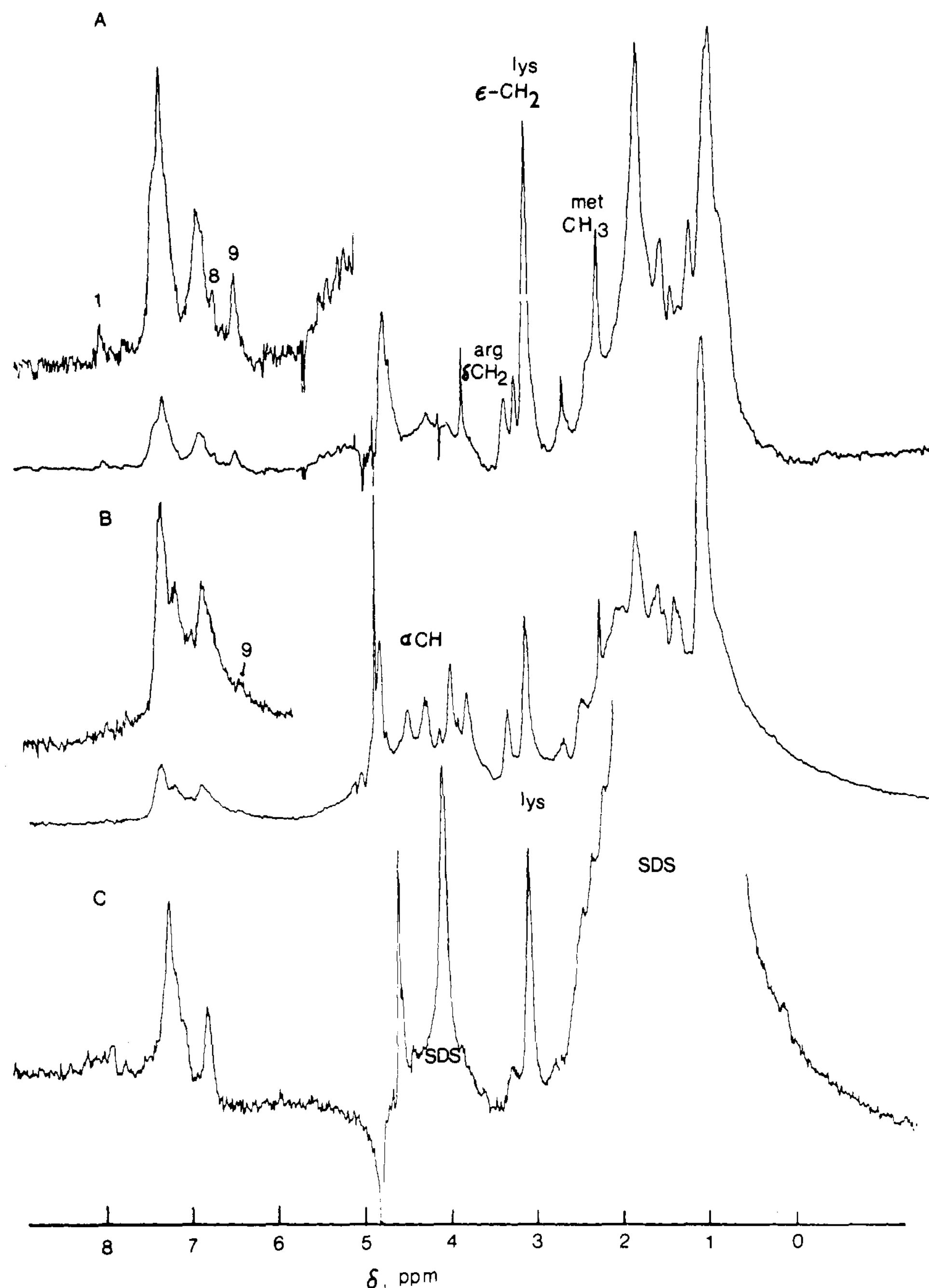

FIGURE 5: The 270-MHz ^1H NMR of gene 5 protein, 1×10^{-3} M, in 0.01 M DPO_4^{2-} pH 8.0 (A); in 0.01 M borate buffer, pH 8.0 (B); in 0.01 M DPO_4^{2-}–0.02 M sodium dodecyl sulfate, pH 8.0 (after boiling).

rotyrosyl residues have chemical shifts similar to that expected for free *m*-fluorotyrosine. The two downfield resonances undergo no detectable change on binding of the tetranucleotide, while the other three shift 0.1 to 0.3 ppm upfield. The middle resonance of the three may not shift resulting in coalescence of two of the closely spaced resonances.

^{31}P NMR of Bound and Unbound Octanucleotide. The ^{31}P NMR spectrum of the tetranucleotides of random sequences in 10 mM phosphate buffer is typical of that for an oligonucleotide with the resonance for the 3′–5′ diester phosphate 2.55 ppm upfield from that for inorganic phosphate, while the 5′-monoester is ~0.5 ppm downfield (Figure 8A). The ^{31}P NMR spectra of tetranucleotides isolated from the limit digest of calf thymus DNA with pancreatic DNase consistently show at least two resonances at chemical-shift values expected for the 5′-monoester group. This may reflect the presence of different bases at the 5′ termini, but might also reflect the presence of some 3′-monophosphates, although pancreatic DNase is presumed to yield only 5′-phosphate end groups. We do not yet have enough data on model nucleotides to be certain of the interpretation.[3] For present purposes this finding does not interfere with the qualitative interpretation of the ^{31}P spectra of the complexes. The homogeneous oligodeoxynucleotides of defined sequence do not show a double resonance in the 5′ position.

[3] Additional spectra show the most upfield of the pair of resonances assigned to the terminal monophosphates to be a doublet, compatible with the proton coupling expected for a 3′ phosphate rather than the triplet expected for a 5′ phosphate.

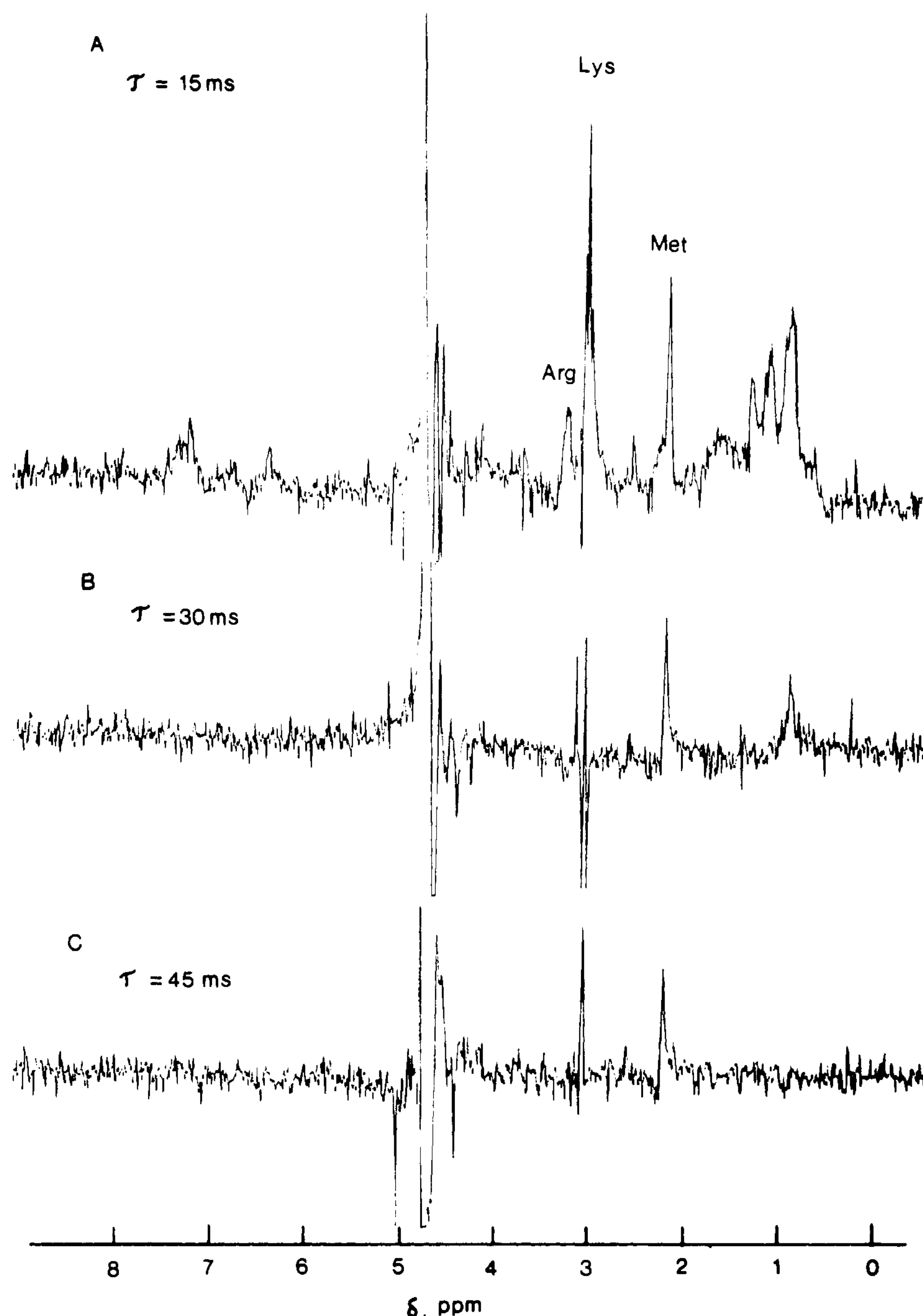

FIGURE 6: The 270-MHz ^{1}H NMR of gene 5 protein, 1.5×10^{-3} M, 0.01 M DPO_4^{2-}, pH 8.0. Carr-Purcell A pulse sequences. (A) τ = 15 ms; (B) τ = 30 ms; (C) τ = 45 ms.

On binding of the random tetranucleotides to gene 5 protein, the resonance of the 3'–5' diester shows a very slight upfield shift (Figure 8B). The ^{31}P NMR spectrum of $d(pT)_8$ in D_2O in the absence of buffer and EDTA is shown in Figure 8C. Paramagnetic contaminants and the lower pH account for the broadness and the slight upfield shift of the resonance for the 5'-phosphates. In the complex of $d(pT)_8$ with gene 5 protein at pH 8, the resonance of the 5'-phosphate is ~1 ppm downfield from that for inorganic phosphate or ~0.5 ppm further downfield than it is in the free nucleotide under the same conditions (Figure 8D). In contrast to the complex with the tetranucleotide, the resonance of the 3'–5' diesters in the octanucleotide complex shows a distinct splitting (Figure 8D).

T_1 for the 3'–5' diester resonance in the free tetranucleotide is 3.5 s, while T_1 for the same resonance in the complex is 1.5 s. T_1 for the resonance of the 3'–5' diester in the octanucleotide is 3.5 s, while T_1 for the same resonance in the complex is 1.8 s. All T_1 determinations were performed on solutions to which 1 mM EDTA had been added.

Discussion

We have constructed a working model of a gene 5 protein-nucleotide complex based on a combination of the NMR data presented here and previous results of chemical modification of the protein and its nucleotide complexes (Anderson et al., 1975). A stereoscopic picture of this model is shown in Figure 9. The first assumption involved in construction of the three-dimensional structure is the prediction of the secondary structure of the protein by the method of Chou and Fasman (1974). The major feature predicted by application of this

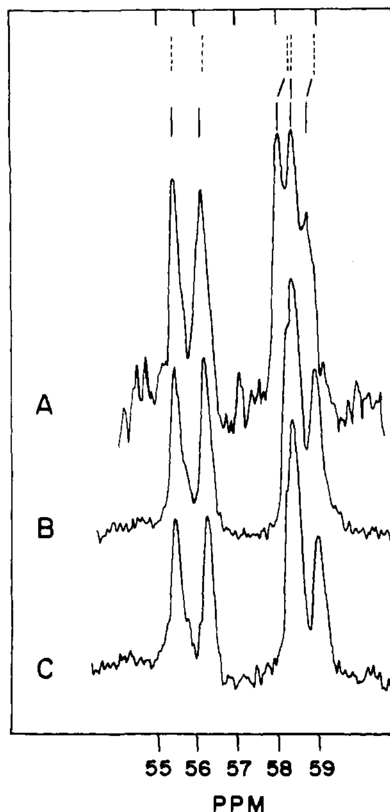

FIGURE 7: ^{19}F NMR of *m*-fluorotyrosyl gene 5 protein, 7.5 × 10^{-4} M (A); plus 1 equiv of tetranucleotides of random sequences, $d(pN)_4$ (B); plus 3 equiv of $d(pN)_4$ (C); the vertical lines represent the chemical-shift positions of the resonances in the free protein (—) and in the tetranucleotide complex (- - -). Conditions: 0.01 M Tris-HCl, pH 8.0, 25 °C.

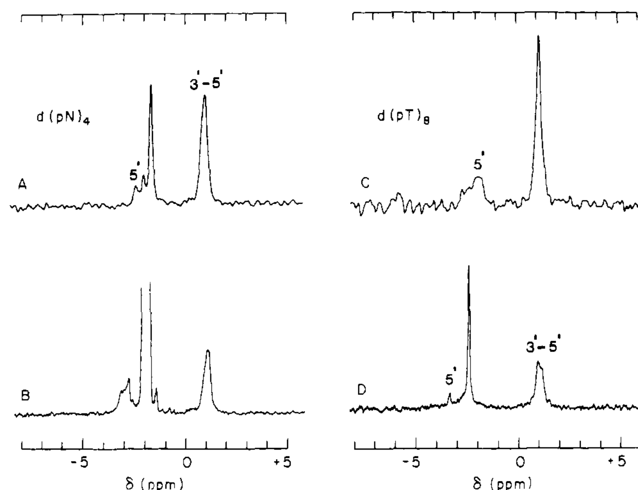

FIGURE 8: ^{31}P NMR of tetradeoxynucleotides, octadeoxynucleotides, and their complexes with gene 5 protein. (A) Tetranucleotides of random sequences, $d(pN)_4$, 3 × 10^{-3} M; (B) gene 5 protein, 1 × 10^{-3} M, plus $d(pN)_4$, 1 × 10^{-3} M; (C) $d(pT)_8$, 2 × 10^{-3} M; (D) gene 5 protein, 2 × 10^{-3} M, plus $d(pT)_8$, 1 × 10^{-3} M. Conditions: 0.01 M HPO_4^{2-}, pH 8.0 (A and D); 0.02 M HPO_4^{2-}, pH 8.0 (B); pH 6.5, no buffer (C); all at 25 °C.

analysis to the sequence of gene 5 protein is the presence of a large percentage of β structure (Anderson et al., 1975). We have arranged these β regions as two large antiparallel β-pleated sheets, one of which wraps horizontally around the rear of the model (residues 60 through 84) and a second (residues 28 through 50) coursing vertically down the right-hand front edge of the model and forming the right boundary of the groove in which the nucleotide binds. While this folding of the predicted secondary structure into a particular tertiary structure must be considered speculative, it is the result of numerous attempts to fold the secondary structure of the molecule into a form that accounts for as many of the known chemical and spectroscopic features of the molecule and its nucleotide complexes as possible. Below we summarize these features and attempt to integrate them with the NMR results.

Chemical modification data have shown that Tyr-26, -41, and -56 lie near the surface of the protein and are readily nitrated by tetranitromethane which prevents DNA binding (Anderson et al., 1975). Tyr-34 and -61 are unavailable for nitration and presumably "buried." Binding of DNA or a tetranucleotide to gene 5 protein prior to reaction prevents nitration of Tyr-26, -41, and -56 (Anderson, 1976). The ^{19}F NMR of the fluorotyrosyl-containing protein suggests the same division of tyrosyl residues (Figure 7). Residues 34 and 61 would appear to be represented by the downfield ^{19}F resonances, the downfield shift relative to free *m*-fluorotyrosine being expected for fluorotyrosyl residues enclosed within protein structure with alteration of the surrounding dielectric constant, more van der Waals contacts and relative immobilization (Hull and Sykes, 1974, 1975). The three resonances grouped near the position expected for free *m*-fluorotyrosine must be the "surface" tyrosyls 26, 41, and 56. We have thus placed Tyr-34 and -61 in the interior of the molecule between the right-hand end of the horizontal β sheet and the upper end of the vertical β sheet (Figure 9).

Optical activity due to the tyrosyl chromophores dominates the CD spectrum of gene 5 protein and the magnitude of the major positive ellipticity band at 228 nm decreases by 25% when the protein binds to DNA (Day, 1973; Anderson et al., 1975). Thus the tyrosyl residues appear to be in an organized array that is altered by nucleotide binding. A number of the tyrosyl proton resonances in the uncomplexed protein occur at 6.49 ppm (Figure 1, Table I) considerably upfield, suggesting some ring current shift (stacking). However, these shifts are not large and the optical activity may be more sensitive to larger stacking distances since rather close stacking distances would be required for the resonances of the aromatic protons to undergo large ring current shifts. From the shifts induced in the ^{19}F NMR spectrum by nucleotide binding (Figure 7), it is the three "surface" tyrosyls that change environment on nucleotide binding. Thus we have folded the structure so as to place Tyr-26, -41, and -56 in the DNA binding groove. The additional specific interaction with the nucleotide is an intercalation of the tyrosyl rings with the base rings to form a continuous stack. In order to illustrate the backbone features of the nucleotide, an octanucleotide, $d(pTpA)_4$, is shown; however, only one bound gene 5 monomer is shown covering bases 4, 5, 6, and 7 from the 5′ terminus at the lower left. Tyr-26 is placed between bases 4 and 5, Tyr-56 between bases 5 and 6, while Tyr-41 intercalates after base 7, leaving bases 6 and 7 stacked together. With adjustment in the arrangement of random sections of the polypeptide chain there is enough variation possible in the placement of the center tyrosyl residue (Tyr-56) to accommodate other stacking arrangements, e.g., intercalation of Tyr-56 between bases 6 and 7.

The strongest evidence for a stacked structure in the complex involving the tyrosyl residues of the protein and the bases of the nucleotide are the upfield shifts of 30 to 40% of the resonances of the aromatic protons when a nucleotide binds (Figures 1, 3, and 4). The chemical shifts of these resonances in the

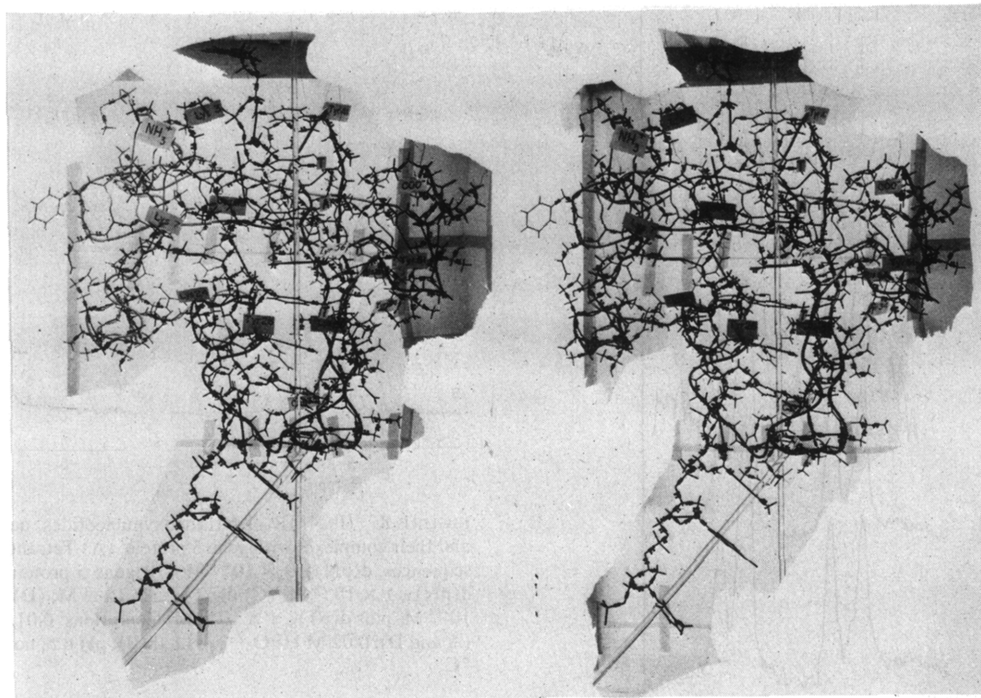

FIGURE 9: Stereoscopic picture of a Kendrew model of a gene 5 protein–d(pTpA)$_4$ complex. The gene 5 monomer covers bases 4, 5, 6, and 7 from the 5′ terminus of the nucleotide at the lower left.

free protein strongly support their assignment to tyrosyl protons (Table I). The relative values of the upfield shifts for the most downfield tyrosyl resonances, ~0.3 ppm when d(pT)$_8$ binds and ~0.8 ppm when d(pA)$_8$ binds, are in agreement with the ring current shifts expected of single protons when placed over pyrimidine and purine rings, respectively (Giessner-Prettre and Pullman, 1970, 1971). Ring current shifts of tyrosyl protons have also been observed on the interaction of tyrosyl-containing peptides with nucleotides (Dimicoli and Hélène, 1974).

In the absence of other perturbing forces, ring current shifts should be independent of the nucleus under observation. Upfield shifts in the ^{19}F resonances of *m*-fluorotyrosyl gene 5 protein from 0.1 to 0.3 ppm are observed on nucleotide bindings (Figure 7). These are of the same magnitude as the upfield shifts observed for some of the aromatic protons on formation of the nucleotide complex (Table I).[4] Only the three ^{19}F resonances identified as originating from surface tyrosyls undergo chemical shifts on nucleotide binding. These findings strongly suggest that the aromatic protons of Tyr-34 and -61 are likely to be unperturbed by nucleotide binding and that the shifts observed in the aromatic region of the proton NMR spectrum can be largely assigned to the 12 protons of Tyr-26, -41, and -56.

The resonance of the C(6)H aromatic proton of thymine shifts upfield by 0.1 to 0.2 ppm in the gene 5 protein–d(pT)$_8$ complex (Figure 1), suggesting that the pyrimidine ring is under the influence of ring currents from the aromatic residues of the protein. The same is suggested by the movements of the resonances of the C(8)H and C(2)H protons of adenine in the d(pA)$_8$ complex (Figure 3), but the shifts are difficult to interpret precisely because of the "unstacking" of d(pA)$_8$ which must accompany complex formation. There are probably several different environments for the thymine ring in the complex since at least two if not more resonances due to the C(6)H appear to be present (Figure 1B). The broadness of the resonance also suggests that the bases are relatively immobilized in the complex which has a minimum molecular weight of 20 000. The latter conclusion is also supported by the significant decrease in T_1 of the phosphorus of the 3′–5′ diester on formation of the complex, suggesting a more rigid structure. While the 5′-terminal phosphorus of the nucleotide experiences a somewhat different chemical environment in the complex (Figure 8), the 3′–5′-phosphate shows little chemical shift in either the tetra- or octanucleotide complexes, suggesting that the conformation of the diester backbone of the nucleotide is not drastically altered from the relatively extended form in the free nucleotide. The split in the resonance of the 3′–5′ phosphates in the octanucleotide complex (Figure 8D) may reflect twist introduced into the nucleotide backbone by the binding of two or more gene 5 monomers.

Octanucleotide binding could conceivably induce formation of longer polymers via the mechanism of one protein binding two bases on each of two adjacent nucleotides. Significant formation of tetramers of gene 5 protein in the presence of d(pT)$_8$ has recently been detected by ultracentrifugation (Cavalieri et al., 1976). Such "mismatched" structures might be stabilized by the protein–protein interactions possible in the octanucleotide complex, but not possible in tetranucleotide complexes. The largest oligomer of gene 5 protein observed in the presence or absence of tetranucleotides was the dimer (see below) (Pretorius et al., 1975; Cavalieri et al., 1976). On the other hand, tetranucleotide binding induces the same shifts in the aromatic resonances as octanucleotide binding. Hence tetranucleotide binding appears to interact with the same specific residues of the protein as longer nucleotides suggesting the same mode of binding for the tetranucleotide. This strongly supports the linear model shown in Figure 9.

High-power electron micrographs of the gene 5 protein–fd DNA complex suggests that some sort of supercoiled structure

[4] Only one of the four possible proton environments on each tyrosyl residue is sampled by the ^{19}F derivative; hence, the ^{1}H and ^{19}F ring current shifts would not be expected to be exactly the same.

is induced in the DNA by the sequential binding of gene 5 monomers (Alberts et al., 1972; Pratt et al., 1974). This conclusion is supported by the fact that the diameter of the complex is ~160 Å, considerably greater than that required by two parallel protein-covered single strands (Pratt et al., 1974). In the model we have chosen to introduce a major twist in the nucleotide backbone at the monomer–monomer interface, as illustrated by the angle between the glass rods in Figure 9. Hence the forces governing supercoiling are contributed by protein–protein interactions. An alternative is to propose that the binding groove of one protein rotates each base relative to its neighbor and, hence, the twist would be evenly distributed along the chain. However, if the particular nature of supercoiling in nucleoprotein structures is controlled predominantly by interactions between the bound proteins for which there is some evidence, then the particular twist must be coupled to these protein–protein interactions. Observation of two different 3′–5′ phosphate resonances in the octanucleotide complex supports the idea that diester conformation is not uniform along the octanucleotide chain in the complex but may be in the tetranucleotide complex (Figure 8).

A number of additional features of the structures of gene 5 protein can be suggested from the 270-MHz ^{1}H NMR spectra. The absence of distinct resonances in the α-CH region of the native protein compared with their presence in the denatured protein (Figure 5), the presence of ring current shifted methyl resonances in the native protein (Figure 1), and the marked change in the resonances of the methyl groups of the aliphatic side chains on denaturation (Figure 5) all suggest that gene 5 proteins must contain a large percentage of fixed structure without large regions of flexible polypeptide chain. This conclusion is also supported by the short T_2 values for most of the resonances (Figure 6). On the other hand, the lysyl and methionyl side chains give rise to narrow resonances with long T_2 values, suggesting that these side chains are on the surface and undergo relatively free rotational motion, compatible with the model (Figure 9) and with previous studies of the acetylation of the lysyl amino groups (Anderson et al., 1975).

A surprising feature of the proton NMR spectra is that the lysyl ϵ-CH_2 protons do not undergo significant chemical shift or line broadening on formation of the nucleotide complexes (Figures 1 and 3), even though acetylation of the lysyl amino groups completely prevents nucleotide binding (Anderson et al., 1975). It may be that the charged ϵ-amino groups provide a neutralizing charge cloud for the negative phosphate backbone of the nucleotide, but do not form highly rigid salt bridges (hydrogen bonds) and hence continue to show a good deal of rotational freedom on the NMR time scale. This may explain the fact that, even in the DNA complex, the lysyl residues remain completely accessible to acetylation (Anderson et al., 1975).

At least two studies of the hydrodynamic properties of gene 5 protein suggest that the homogenous protein exists primarily as the dimer (Pretorius et al., 1975; Cavalieri et al., 1976). It is difficult to be certain from the NMR line widths whether the spectrum represents the dimer or monomer, but the lines are broad enough to be not incompatible with the rotational correlation time of the dimer. In any event the spectrum of the $d(pT)_8$ complex shows considerable broadening compared with the spectrum of either the free protein or the tetranucleotide complex (Figure 1). Induction of tetramer formation by $d(pT)_8$ has recently been suggested by equilibrium ultracentrifugation (Cavalieri et al., 1976). While some of the broadening in the ^{1}H NMR of the complex may be due to formation of higher oligomers as discussed above, the marked changes in the resonances of the aliphatic methyl groups on formation of the octanucleotide complex (Figure 1) compared with the tetranucleotide complexes probably reflect hydrophobic interactions between adjacent monomers tied together by the covalent structure of the nucleotide and characteristic of the protein–DNA complex.

In the model the single sulfhydryl group (Cys-33) has been placed at the base of the DNA-binding groove. The position of this residue is compatible with the finding that nucleotide binding prevents access of Hg^{2+} to the -SH group (Anderson et al., 1975) and with the finding that thymidine residues in nucleotide complexes of gene 5 protein can be cross-linked to the cysteinyl residue by ultraviolet irradiation (Nakashima and Konigsberg, 1975).

At present we have not specifically brought any of the four arginyl residues into contact with the phosphate backbone, although all occur close to the surface. While these may be involved in protein–protein interactions, at least two of the guanidino groups could be brought near the phosphate backbone by alteration in stretching of the polypeptide chain not specifically structured by the Chou–Fasman analysis. Resonances from the δ-CH_2 groups of the arginyl residues clearly undergo chemical shifts, broadening, or both on tetranucleotide and octanucleotide binding (Figures 1 and 3). While this may reflect conformational change in the surface of the protein, it could represent direct interaction of the guanidino groups with the phosphate backbone, although the changes in these resonances are not identical with the two octanucleotides.

There are significant regions of the polypeptide chain for which not enough information exists to fix them specifically in the model. Only general principles have been applied. As many as possible of the hydrophobic side chains have been directed to the interior of the molecule while maintaining charged side chains on the surface. A number of hydrophobic side chains must be on the surface since the protein contains a particularly high percentage of hydrophobic residues which probably accounts for its tendency to precipitate at concentrations of 20 to 30 mg/ml. The DNA-binding groove is lined by many hydrophobic side chains, and van der Waals contacts, hydrophobic interactions, and accompanying dehydration of the DNA may account for many of the binding forces. However, a number of polar side chains, particularly serine and threonine, are in close proximity to the DNA-binding groove. We have no evidence as yet for hydrogen bonding between the bases and protein side chains, but such hydrogen bonding may occur and could account for the differential binding affinity of nucleotides as a function of base sequence (Figure 2). The fact that this protein can distinguish base sequences by alterations in binding constants of $\sim 10^2$ (Figure 2) has possible implications for the function of this and similar proteins since it could determine initial sites of binding and, hence, nucleation sites from which the complex is propagated. DNA-binding proteins with such differential binding affinity for the single strand could also direct the melting of specific regions of double-stranded DNA.

We would like to call attention to some general features of the model where variation in structure is possible. In the initial formation of the hydrogen bonds of the β-pleated sheets, we have chosen to fold sequential sections of the β-structured polypeptide chain back on each other. In both cases rather typical β turns can be constructed involving residues 40 to 43 (Glu-Tyr-Pro-Val) and 71 to 74 (Gly-Gln-Phe-Gly). The first region of β sheet from the N-terminal end, that lying vertically along the DNA-binding groove, contains the intercalating

Tyr-41. Fixation of this residue at the upper end of the DNA-binding groove makes alternative matching of the β chains awkward, but it might be accomplished if sufficient twist or folding were introduced. On the other hand, the second long section of β-pleated sheet contains no residues with the possible exception of Lys-69 which potentially interacts with DNA on the surface of the molecule. We have thus wrapped this sheet around the rear of the molecule in a barrel-like structure. There is considerable latitude in the placement of this sheet and it can be twisted or moved up and down without altering the model in major fashion. It must be folded or broken in some fashion unless the molecule is elongated. We have chosen to pack the side chains as compactly as possible which in the current model results in a molecule approximately 35 × 27 × 20 Å. The DNA-binding groove and the repeating unit of four nucleotide residues spans 24 Å compared with 28 Å expected for a completely extended tetranucleotide. A significant amount of overlap between two gene 5 monomers may occur in the oligomeric nucleotide complexes.

Acknowledgments

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