A 500-MHz Proton Nuclear Magnetic Resonance Study of the Structure and Structural Alterations of Gene-5 Protein–Oligo(deoxyadenylic acid) Complexes†

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ABSTRACT: The complex of the gene-5 protein of bacteriophage M13 with octadeoxyadenylic acid [$d(A)_8$] has been shown earlier to differ in various respects from the complex with polynucleotides [Alma, N. C. M., Harmsen, B. J. M., van Boom, J. H., van der Marel, G., & Hilbers, C. W. (1982) *Eur. J. Biochem. 122*, 319–326]. In this paper the gene-5 protein–$d(A)_8$ complex is compared with the complex formation between the gene-5 protein and a mixture of longer oligonucleotides, i.e., $d(A)_{25-30}$. Nuclear Overhauser experiments have been performed on both systems to obtain structural information regarding the oligonucleotide protein interactions. In the experiments also oligonucleotides deuterated at the adenyl C_8 positions have been used in order to distinguish between the adenyl H_2 and H_8 resonances. Combination of the experiments shows that the nucleotides in the complexes are situated in such a way that the adenyl H_8 and the sugar $H_{1'}$ protons are near the protein surface while the adenyl H_2 protons are relatively far removed from all other protons, indicating that this side of the base is pointing away from the protein surface. It is concluded that structurally different complexes can be obtained for the $d(A)_{25-30}$ system. The complex with $d(A)_{25-30}$ undergoes a structural transition when going from excess oligonucleotide to excess gene-5 protein. This transition is identified with the transition between the "oligonucleotide" and the "polynucleotide" binding mode. The information derived from the present NMR experiments combined with known data from X-ray diffraction and electron microscopic studies is used to propose a model for a possible orientation of the adenyl bases in the complex.

The gene-5 protein encoded by bacteriophages M13, fd, and f1 is a single-stranded DNA[1] binding protein belonging to the group of so-called DNA helix destabilizing proteins. It is synthesized in great amounts in the infected *Escherichia coli* cell, where it is required for single-stranded viral DNA synthesis (Alberts et al., 1972; Oey & Knippers, 1972; Ray, 1977). The DNA binding process in which a protein monomer covers approximately four nucleotide units (Alberts et al., 1972; Pretorius et al., 1975; Cavalieri et al., 1976; Day, 1973) is strongly cooperative.

The gene-5 protein has a molecular weight of 9690 as determined from the amino acid sequence (Cuyper et al., 1974; Nakashima et al., 1974a,b) and exists in solution predominantly as a dimer (Pretorius et al., 1975; Cavalieri et al., 1976). Also in crystallographic studies the protein is observed as a dimer. The presumed DNA binding groove is 30 Å in length, having opposite polarity in the two halves of the dimer. Of the aromatic residues tyrosines-26, -34, -41, and -56 and phenylalanines-13 and -68 may be present in the DNA binding groove. In addition lysines-24 and -46 and arginines-21, -80, and -82 are in a position to interact with the DNA (McPherson et al., 1980a). X-ray diffraction studies of gene-5 protein cocrystallized with small oligonucleotides suggest that the complex is a disklike aggregate consisting of six protein dimers arranged in a circle (McPherson et al., 1980a,b).

In the electron microscope the gene-5 protein–fd DNA complexes are observed as helical rods with a width of 100 Å and a mean helical pitch of 91 Å (Torbet et al., 1981; Gray et al., 1982). Also NMR experiments have provided information about the complex formation between the gene-5 protein and oligonucleotides (Coleman et al., 1976; Garssen et al., 1977, 1978, 1980; Coleman & Armitage, 1978; Alma et al., 1981a). Recently we have concluded from NMR studies that one to two lysines and two to three arginines are immobilized upon complex formation (Alma et al., 1981b) in correspondence with suggestions from the X-ray data (McPherson et al., 1980a). In addition we have shown that the gene-5 protein covers approximately three nucleotide residues when complexed with the oligonucleotides $d(A)_8$, $d(A)_{12}$, or $d(A)_{16}$ (Alma et al., 1982). This is in contrast with the stoichiometry of approximately four nucleotides per protein found for the complex with polynucleotides. Hence, after the length of the oligonucleotide is increased, a transition must be found between the two binding modes. In this paper we will compare the complex formed between the gene-5 protein and $d(A)_8$ with a complex of the gene-5 protein with a mixture of longer oligonucleotides, $d(A)_{25-30}$. We will show that for the latter complex a transition between two different binding modes can be observed. These complexes are further examined by employing the nuclear Overhauser effect. In previous nuclear Overhauser experiments we were not able to distinguish between H_8 and H_2 adenyl resonances (Alma et al., 1981a). By making use of oligonucleotides deuterated at the adenyl C_8 positions, the H_2 and H_8 resonances can be identified. This enables us to derive additional information about the structure of the gene-5 protein oligonucleotide complexes with the emphasis on the oligonucleotide structure. A model for the orientation of the bases is proposed which shows that double

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; DNA, deoxyribonucleic acid; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; FID, free induction decay; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, parts per million; RD, relaxation delay.

0006-2960/83/0422-2104$01.50/0

helix formation is no longer possible.

Materials and Methods

Materials. The isolation and purification of the gene-5 protein were essentially as described previously (Garssen et al., 1977). The octanucleotide $d(A)_8$ was synthesized by using the phosphotriester method (Arentzen et al., 1979). $d(A)_{25-30}$, a mixture of oligo(deoxyadenylic acids) ranging in length from 25 to 30 residues, was purchased from P-L Biochemicals. Oligonucleotides were used as the Na^+ salts. Excess salt was removed by passage over a Sephadex G-10 column. In spite of this, the $d(A)_{25-30}$ sample contained some impurities. These impurities did not interfere in the binding, and their resonances have been removed from the spectra as indicated in the figures. For NMR measurements the oligonucleotides were lyophilized 3 times from 99.75% D_2O. Replacement of the adenyl H_8 protons with deuterons was accomplished by incubation during 8 h at neutral pH and 80 °C. All NMR samples were contained in 99.75% D_2O. Concentrations were determined from UV absorption by using the extinction coefficients ϵ_{276nm} = 7100 M^{-1} cm^{-1} for the gene-5 protein (Day, 1973), ϵ_{260nm} = 82 600 M^{-1} cm^{-1} for $d(A)_8$, and ϵ_{260nm} = 9500 M^{-1} cm^{-1} for $d(A)_{25-30}$ (in mononucleotides). The extinction coefficients of the oligonucleotides were determined by enzymatic degradation of the oligonucleotides with venom phosphodiesterase (Worthington) (Cassani & Bollum, 1969) by using an extinction coefficient for AMP of 15 300 M^{-1} cm^{-1}. The concentration of $d(A)_{25-30}$ is given in moles of mononucleotide per liter.

Instrumentation. Ultraviolet absorption measurements were carried out with a Zeiss PMQII spectrophotometer. The pHs reported are the uncorrected pH meter readings in D_2O solutions. ^1H NMR spectra (500 MHz) were recorded on a Bruker WM-500 spectrometer operating in the Fourier transform mode. ^1H NMR (360 MHz) spectra of $d(A)_8$ were recorded on a Bruker HX-360 spectrometer. Chemical shifts are quoted relative to DSS. Downfield shifts are defined as positive. Spectra were resolution enhanced by a Lorentzian to Gaussian transformation (Ferrige & Lindon, 1978).

Nuclear Overhauser experiments were performed by employing the pulse sequence $[(RD–Dec(t_1,\omega_A)–FID(+)–RD–Dec(t_1,\omega_{off})–FID(-)]_n$. After a relaxation delay (RD) (1–2 s) a selective presaturation pulse of duration t_1 is applied by the ^1H decoupler at a chosen resonance A with resonance frequency ω_A. This is followed by a nonselective observation pulse, and the resultant FID is recorded. After a second relaxation delay a presaturation pulse of the same duration t_1 is applied at an off-resonance position ω_{off} (at the edge of the spectrum). Subtraction of the second FID from the first yields the NOE difference FID. The sequence is repeated appropriately for the desired signal to noise ratio. The nuclear Overhauser effect (NOE) originates as a result of coupled dipolar relaxation (cross relaxation) of neighboring spins. For a two-spin system the dipolar cross relaxation consists of zero (flip-flop) and double quantum transitions. For the systems considered in this paper the flip-flop term predominates. Because more than two spins interact in these systems, the flip-flop term gives also rise to spin-diffusion effects. These spin-diffusion effects can be minimized by shortening the preirradiation time.

All measured NOE's were negative, but transformed NOE difference spectra were phased so as to give positive peaks. NOE difference spectra were sensitivity enhanced by multiplication of the FID with a negative exponential resulting in a line broadening of 4 and 10 Hz in the NOE experiments performed with the gene-5 protein–$d(A)_8$ complex and the gene-5 protein–$d(A)_{25-30}$ complex, respectively. Unless indicated otherwise all spectra were recorded at 28 °C.

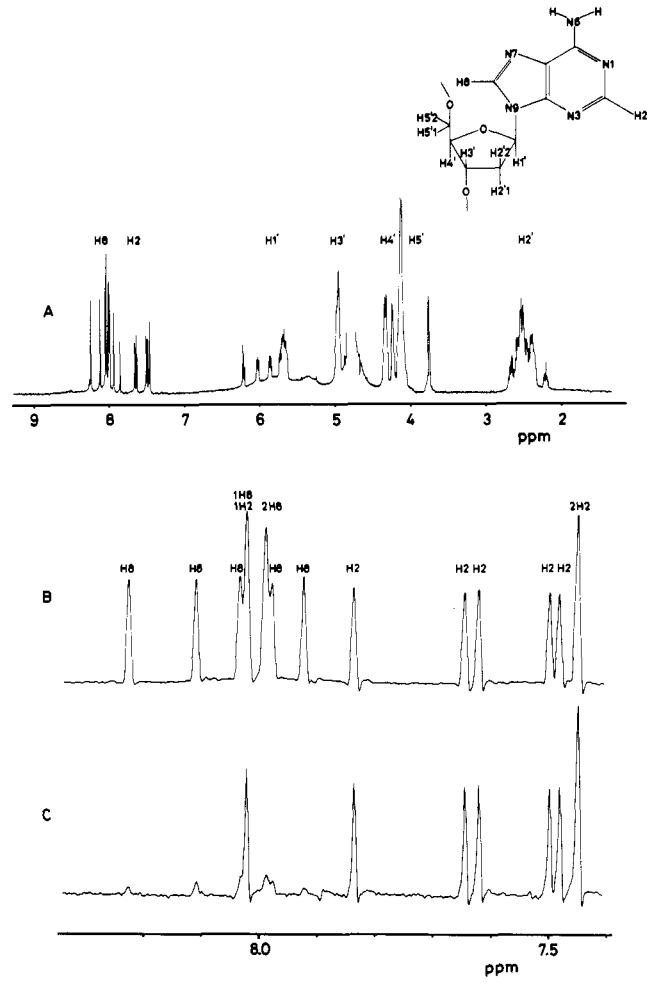

FIGURE 1: (A) 500-MHz ^1H NMR spectrum of 1.2 mM $d(A)_8$. (B) Aromatic region of the 360-MHz ^1H NMR spectrum of 4 mM $d(A)_8$. (C) Aromatic region of the 360-MHz ^1H NMR spectrum of 3 mM H_8-exchanged $d(A)_8$. The spectra were recorded at 30 °C. The assignments of the resonances to H_8 and H_2 protons are given in (B). The inset in (A) shows the numbering of the atoms in a nucleotide unit.

Ring current induced shifts of the adenyl H_2 and H_8 protons were calculated by the methods of Giessner-Prettre & Pullman (1970; Giessner-Prettre et al., 1976). In these calculations the shifts caused by the five- and six-membered rings of the two nearest-neighbor and the two next nearest neighbor adenines in the helical structure were taken into account. The atomic coordinates of the adenyl bases used in these calculations were taken from Arnott (Arnott & Hukins, 1972). To obtain agreement between calculated and measured shifts, we varied the positions and orientation of the adenine rings within the constraints dictated by electron microscopy experiments (Torbet et al., 1981) (see Discussion).

Results

Spectrum of $d(A)_8$ and of Its Complex with the Gene-5 Protein. In Figure 1A the 500-MHz ^1H NMR spectrum of $d(A)_8$ is given. The assignment of the resonances to the different kinds of protons is based on the chemical shifts known from the literature (Olsthoorn et al., 1980). The numbering of the protons is shown in the inset in Figure 1A.

The resonances of chemically equivalent protons on different nucleotide units, e.g., the $H_{1'}$ protons, do not coincide, indicating that the oligonucleotide does not have an averaged

Table I: Resonance Positions of the H_2 and H_8 Protons of $d(A)_8$ Free in Solution (δ_{free}) and of $d(A)_8$ Which Is Partly Complexed with Gene-5 Protein (δ_{exp})

assignment	δ_{free}		δ_{exp}	resonance label[a]	$\Delta\delta$ [c]
H_8	8.223		8.197	a1	−0.026
H_8	8.108		8.057	a2	−0.051
H_8	8.031	[b]	8.030	a3	(−0.001)[b]
H_8	8.019		8.030	a3	(+0.011)[b]
H_2	8.019		8.000	a4	(−0.019)[b]
$2H_8$	7.987		7.953	a5	(−0.034)[b]
H_8	7.975		7.912	a6	(−0.063)[b]
H_2	7.835		7.874	a7	+0.039
H_8	7.919		7.780	a8	−0.135
H_2	7.643		7.748	a9	+0.105
H_2	7.621		7.705	a10	+0.084
H_2	7.497		7.673	a11	+0.176
H_2	7.480		7.646	a12	+0.166
$2H_2$	7.448		7.628	a13	+0.180

[a] Peak labels as shown in Figure 2A. [b] The correspondence between the resonances in the free and the partly complexed state cannot be determined unambiguously in this region; therefore, $\Delta\delta$'s have been placed in parentheses. [c] $\Delta\delta = \delta_{exp} - \delta_{free}$.

regular structure. The $H_{1'}$ resonances at 5.8–6.3 ppm that are resolved from the main $H_{1'}$ peak at 5.7 ppm can be assigned to terminal nucleotide residues, the lowest field resonance at 6.23 ppm belonging to the 3′-terminal nucleotide (Olsthoorn et al., 1980).

An analogous conclusion can be drawn for other protons as well. $H_{2'}$ proton resonances overlap between 2.3 and 2.6 ppm. The $H_{2'}$ resonances at 2.20 ppm that are resolved from the others can be assigned to the 5′-terminal $H_{2'2}$ proton (Olsthoorn et al., 1980). Also at 2.65 ppm a resolved resonance appears probably belonging to the 3′-terminal $H_{2'2}$ proton (vide infra).

The upfield shifted $H_{5'}$ resonance at 3.76 ppm belongs to the 5′-terminal nucleotide residue.

The resonances of H_2 and H_8 protons fall between 7.4 and 8.3 ppm. A spectrum was recorded of $d(A)_8$ in which the adenyl H_8 protons were replaced by deuterons to be able to distinguish between the H_8 and H_2 resonances. Below, we will denote this material as H_8-exchanged $d(A)_8$. In Figure 1B,C the aromatic regions of the 360-MHz ^1H NMR spectra of $d(A)_8$ and H_8-exchanged $d(A)_8$ are presented. Comparison of the two spectra allows assignment of the resonances to H_8 and H_2 protons as indicated above the spectrum in Figure 1B.

The spectra of the complexes of gene-5 protein in the presence of excess $d(A)_8$ and excess H_8-exchanged $d(A)_8$ are given in parts A and B of Figure 2, respectively. Again by comparison of the two spectra resonances can be assigned to H_8 or H_2 protons.

The chemical shifts of the H_2 and H_8 proton resonances in Figure 2A are listed in Table I together with the chemical shifts of these protons in the free state. The assignment of a resonance in the spectrum of the complex to the same H_8 and H_2 proton is in accordance with earlier titration experiments (Alma et al., 1982) in which the resonances were followed as a function of the ratio $d(A)_8$:protein. We note in passing that there is fast exchange for $d(A)_8$ free in solution and complexed to the protein. Because of the severe overlap and small changes in chemical shift of the H_8 resonances between 8.03 and 7.9 ppm, the correspondence between the resonances in the free and the (partly) complexed state cannot be determined unambiguously. In Table I these resonances have been arranged in order of their chemical shift. Interestingly, Figure 2 shows that the adenyl resonances have undergone differential broadening. The implications of the observed broadening and shifts of the resonances for the structure of the complex are considered under Discussion.

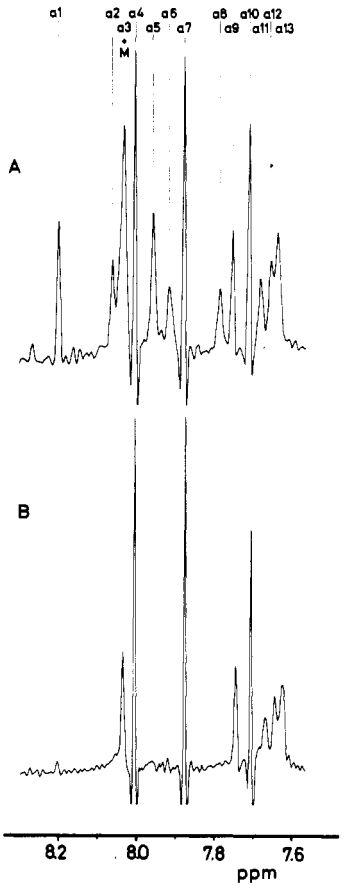

FIGURE 2: Comparison of the spectral regions between 8.1 and 7.6 ppm of the 500-MHz ^1H NMR spectra of the complex of gene-5 protein (1 mM) with excess $d(A)_8$ (0.6 mM) (A) and with excess H_8-exchanged $d(A)_8$ (0.6 mM) (B). [Note that one $d(A)_8$ molecule can bind three protein molecules (Alma et al., 1982)]. The H_2 and H_8 resonances have been labeled a1 to a13 in the gene-5 protein. Resonance M belongs to the C_2 proton of the single histidine. All samples contained 50 mM NaCl and 1 mM cacodylate (pH 7.2). The small differences in chemical shift of several of the equivalent peaks in the two spectra are caused by a small difference in the fraction free $d(A)_8$.

Spectrum of $d(A)_{25-30}$ and of Its Complex with the Gene-5 Protein. The 500-MHz ^1H NMR spectrum of $d(A)_{25-30}$ is given in Figure 3A. The resonances of most of the chemically equivalent protons on different nucleotide units, e.g., H_2 or H_8 protons, coincide at the same spectral position, indicating that the major part of the molecule folds into a regular structure which is probably B-DNA-like (Olsthoorn et al., 1980). The small resonances that are resolved from the major resonances belong to protons in residues to the ends of the oligomer; e.g., the most downfield shifted $H_{1'}$ proton resonance at 6.13 ppm can be assigned to the 3′-terminal residue.

The $H_{5'1}$ and $H_{5'2}$ resonances coincide at 3.98 ppm while the $H_{2'1}$ and $H_{2'2}$ resonances are resolved from each other at 2.30 and 2.45 ppm. The resonance of the 3′ protons is obscured by the HDO signal at our experimental conditions. The resonance positions of the H_2 (7.37 ppm) and H_8 protons (7.92 ppm) indicate that the structure of the oligonucleotide is not completely the same as the structure of poly(dA) in the poly(dT)·poly(dA) double helix where the H_2 and H_8 protons resonate at 6.88 and 7.80 ppm, respectively (Early et al., 1977); the complementary strand is expected to only marginally influence the chemical shift (Giessner-Prettre et al., 1976; Arter & Schmidt, 1976).

Table II: Selective NOE's in the 500-MHz ^{1}H NMR Spectrum of $d(A)_{25-30}$[a]

obsd resonance	preirradiation time	irradiated resonance						
		H_8	H_2	$H_{1'}$	$H_{4'}$	$H_{5'}$	$H_{2'1}$	$H_{2'2}$
H_8	0.15	1		0.07			0.11	0.14
	0.4	1		0.13	0.03	0.06	0.22	0.28
H_2	0.15		1					
	0.4		1	0.04		0.02		
$H_{1'}$	0.15	0.08		1	0.05	0.08	0.13	0.10
	0.4	0.23	0.05	1	0.06	0.12	0.24	0.24
$H_{4'}$	0.15			0.07	1	0.21		
	0.4	0.10	0.01	0.08	1	0.26	0.06	0.18
$H_{5'}$	0.15			0.08	0.13	2		0.14
	0.4	0.14	0.04	0.16	0.27	2	0.11	0.33
$H_{2'}$	0.15	0.14		0.15		0.05	1	0.23
	0.4	0.30		0.19	0.06	0.10	1	0.39
$H_{2'2}$	0.15	0.15		0.06			0.16	1
	0.4	0.35		0.12	0.04		0.32	1

[a] The assignment of the resonances is shown in Figure 3A. The listed values of the NOE's are equal to [(area of observed peak in difference spectrum)/(area of irradiated peak in difference spectrum)] × (number of protons irradiated).

Additional structural information can be obtained by performing NOE experiments. The results of the time-dependent NOE experiments performed with this oligonucleotide are listed in Table II.

The expected helical structure of the oligonucleotide (Olsthoorn et al., 1980) will give rise to an anisotropic rotational correlation time, which makes a quantitative discussion of the observed NOE's not feasible. However, qualitative information can be deduced from these experiments. On the basis of the NOE's observed between the $H_{1'}$ and $H_{2'}$ protons the lower field $H_{2'}$ resonance is assigned to the $H_{2'1}$ and that at higher field to the $H_{2'2}$ proton (see structure in Figure 1A). After 0.15-s preirradiation of $H_{2'2}$ no NOE is observed at the $H_{4'}$ position, whereas after 0.4 s a relatively large NOE is observed at this position. This can be understood when it is realized that magnetization may be first transferred to the (not observable) $H_{3'}$ proton and from there to the $H_{4'}$ proton. After preirradiation of a H_8 resonance a strong NOE is expected for the $H_{2'2}$ proton of the same nucleotide residue and another strong NOE for the $H_{2'1}$ proton of the adjacent nucleotide residue at the 5'-position when the oligomer is in a B-DNA-like conformation. Hence, the observation of strong NOE's for both $H_{2'}$ protons is compatible with the presumed B-DNA-like structure of this oligomer. The small NOE's observed after preirradiation of the H_2 resonance, meaning that the H_2 protons are far removed from the other protons, are also compatible with a B-DNA-like structure. Below we will compare the NOE's observed in the free oligonucleotide with those observed for the gene-5 protein–$d(A)_{25-30}$ complex.

When $d(A)_{25-30}$ is added in excess to the gene-5 protein, a high molecular weight complex (estimated weight ~140 000) is formed. This is illustrated by the broadness of the resonances in the spectrum of this complex (Figure 3B). Comparison of this spectrum with that of the free oligonucleotide reveals that resonances of the free oligonucleotide are visible at their original positions (indicated by the lines drawn between Figure 3A,B). Separately additional nucleotide resonances of the $d(A)_{25-30}$ present in the protein–DNA complex are visible in the spectrum. This is more clearly seen in the extended form of the aromatic part of the spectrum presented in Figure 4B. The resonances belonging to the oligonucleotide are lettered aa1 to aa5. From comparison of Figure 3A,B resonance aa5 is assigned to the $H_{1'}$ proton, resonance aa3 to the H_2 protons, and (part of) resonance aa1 to the H_8 protons of the free oligonucleotide. Assignments of resonances aa2 and aa4 are obtained with the aid of Figure 4C. The spectrum in Figure 4C is that of a complex formed by the protein and

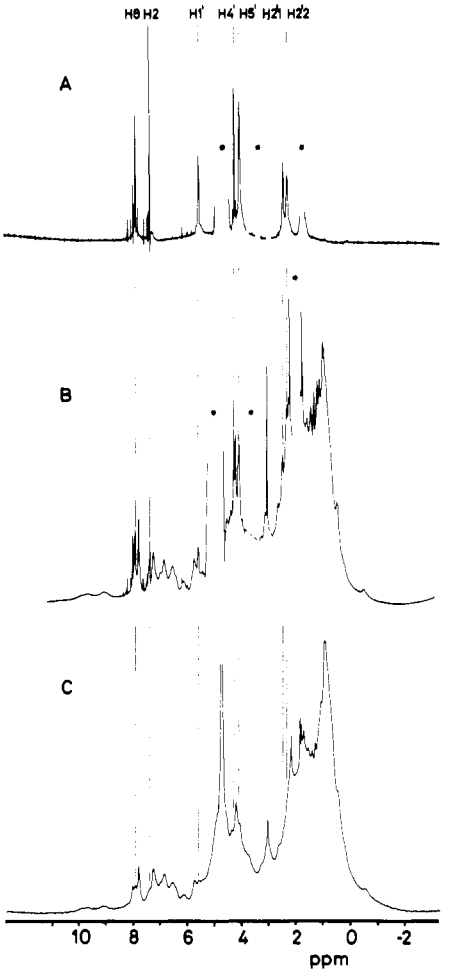

FIGURE 3: (A) 500-MHz ^{1}H NMR spectrum of 5 mM $d(A)_{25-30}$ in 5 mM cacodylate, pH 6.7. (B) 500-MHz ^{1}H NMR spectrum of gene-5 protein (1 mM) with excess $d(A)_{25-30}$ (~6 mM) in 50 mM NaCl and 6 mM cacodylate. (C) 500-MHz ^{1}H NMR spectrum of the gene-5 protein $d(A)_{25-30}$ complex from the same sample as used for the spectrum in (B). Signals of excess $d(A)_{25-30}$ were suppressed by making use of spin diffusion (see text). This spectrum has not been resolution enhanced. The asterisks indicate peaks that have been removed from the spectra: the HDO peak (at ~4.7 ppm), the cacodylate resonance (at ~1.7 ppm), and impurities from the $d(A)_{25-30}$ sample. The spectral positions of the resonances of the free oligonucleotide are indicated by vertical lines.

H_8-exchanged $d(A)_{25-30}$. In the latter case only a slight excess of the oligonucleotide is present. Therefore the resonance at

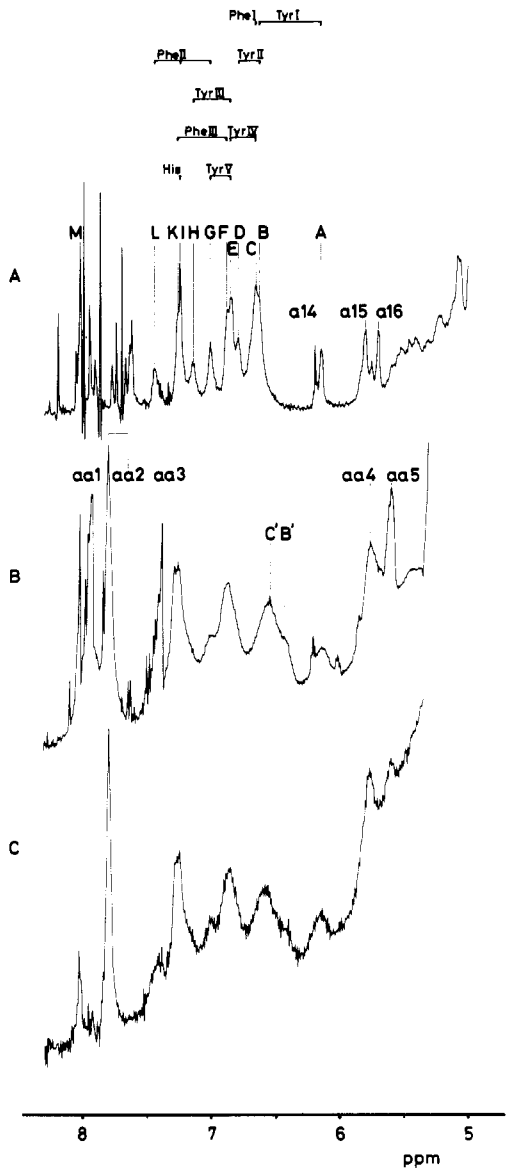

FIGURE 4: Comparison of the spectral regions between 8.3 and 5.0 ppm of complexes which gene-5 protein forms with $d(A)_8$, $d(A)_{25-30}$, and H_8-exchanged $d(A)_{25-30}$. (A) Spectrum of the gene-5 protein–$d(A)_8$ complex; the region between 8.2 and 7 ppm is the same as that shown in Figure 2A. The peaks belonging to protein resonances have been labeled A–M in accordance with former experiments (Alma et al., 1981a). The assignments of the peaks are given above the spectrum. Supplementary to the labels in Figure 2 $H_{1'}$ resonances have been labeled a14–a16. (B) Spectrum of the gene-5 protein–$d(A)_{25-30}$ complex, the aromatic region of Figure 3B. The protein resonances that deviate from the resonances in (A) are labeled B′ and C′. The resonances originating from the oligonucleotide are labeled aa1 to aa5. (C) Spectrum of the gene-5 protein (1.0 mM) with H_8-exchanged $d(A)_{25-30}$ (~4 mM) in 50 mM NaCl and 6 mM cacodylate (pH 6.9).

7.78 ppm (aa2) comes from the adenyl H_2 protons and aa4 at 5.76 ppm from the $H_{1'}$ protons of the oligonucleotide in the bound state. Note that the resonance at 8.03 ppm is from the C_2 proton of His-64 of the gene-5 protein. It is interesting that the adenyl H_2 resonance in Figure 4C is relatively narrow. Moreover, the signals from the terminal H_2 resonances visible in the spectrum of the free oligonucleotide are now clustered at this position. This indicates that all H_2 protons are in the same magnetic environment which in turn indicates that the oligonucleotide has a regular structure in the protein–DNA complex. Furthermore complex formation causes a downfield shift of 0.435 ppm for most of the adenyl H_2 resonances, indicating that in the complex the adenyl bases are less stacked than in the free nucleotide. The situation for the adenyl H_8 resonances is different. This can be demonstrated by a comparison of parts A and B of Figure 3. As described already (vide supra) the spectrum in Figure 3B depicts oligonucleotide resonances from the complex as well as from the free oligonucleotide. The resonances that belong to the complex can be distinguished from those of the free oligonucleotide in an experiment that makes use of the strong spin diffusion in the high molecular weight complex. For this experiment the same pulse sequence was used as for an NOE experiment (see Materials and Methods) except that a preirradiation pulse of relatively high power and long duration (2 s) was used. It was applied at 1.0 ppm where no protons of the oligonucleotide resonate. The resonances that belong to the complex will then become saturated as a result of strong spin diffusion. The resonances belonging to the free oligonucleotide are only influenced to a small extent by saturation transfer, caused by the slow exchange between the free and the complexed states. The spectrum recorded immediately after the preirradiation pulse therefore only contains those resonances that have not been saturated. Subsequent subtraction of this spectrum from the reference spectrum yields the spectrum shown in Figure 3C. Comparison with Figure 3A,B shows that virtually only the resonances of the free oligonucleotide have disappeared in Figure 3C. The resonances of the $H_{2'}$, $H_{4'}$, and $H_{5'}$ protons of the complexed oligonucleotide are obscured by protein resonances. The resonances of the $H_{1'}$ protons can be observed in Figure 3C at 5.76 ppm (aa4 in Figure 4B). As in Figure 4C the adenyl H_2 resonance is at 7.78 ppm. The broad resonances downfield from this peak are from the adenyl H_8 protons. Note that the C_2 proton resonance of His-64 is superimposed on this signal. A difference spectrum of Figure 4B,C (not shown) suggests that the H_8 resonances from the complexed $d(A)_8$ are spread out between 7.8 and 8.0 ppm. As will be discussed below this spread in adenyl H_8 resonance positions is probably caused by the different shifts originating from the stacking of the tyrosyl and phenylalanyl rings upon the adenyl rings. It does not invalidate the conclusion that the structure of the oligonucleotide is regular in the complex.

We now return to Figure 4 where the effect of complex formation between $d(A)_{25-30}$ and the protein on the aromatic part of the spectrum (Figure 4B) is compared with that for $d(A)_8$–protein complex formation (Figure 4A). In both cases excess of oligonucleotide was present. The assignment of the H_2 and H_8 adenyl resonances in Figure 4A has been presented already in Figure 2. The assignment of the protein resonances, based on previous NOE studies (Alma et al., 1981a), is given above Figure 4A. The resonances belonging to Phe I, Tyr I, and Tyr IV undergo large upfield shifts upon binding of the oligonucleotide while small shifts have been observed for the resonance of Phe III.

We first note that the exchange of $d(A)_8$ between the free and the complexed state is fast on the NMR time scale while that of $d(A)_{25-30}$ is slow. The decreased dissociation rate constant in the latter case reflects enhanced cooperativity of the binding of the protein to $d(A)_{25-30}$. The aromatic part of the spectrum of the complexed protein in the $d(A)_{25-30}$–gene-5 protein complex (Figure 4B) is apart from the increased line widths and the resonances C′ and B′ quite similar to that of the $d(A)_8$ complex. In the gene-5 protein–$d(A)_8$ complex peaks B and C contain the ring proton resonances of Phe I, Tyr I, Tyr II, and Tyr IV (see Figure 4A). A considerable amount of the intensity comprised by these resonances has shifted upfield by an amount of ~0.15 ppm, giving rise to resonance

C′B′ in the spectrum of the gene-5 protein–$d(A)_{25-30}$ complex. This shift suggests that $d(A)_{25-30}$ binds in a somewhat different manner to the protein than $d(A)_8$. Peak A belonging to the 3,5 protons of Tyr I remains at the same spectral position. Hence it is unlikely that the 2,6 protons of Tyr I contribute to the observed shift. Also the 3,5 resonances of Tyr II are unlikely to shift since these resonances have remained unaffected by the binding of $d(A)_8$ (Alma et al., 1981a). Therefore we tentatively conclude that the ring proton resonances of Phe I and probably the resonances of the 3,5 protons of Tyr IV are responsible for the shift.

NOE Experiments with the Gene-5 Protein–$d(A)_8$ Complex. Previously we have described NOE experiments carried out to assign the aromatic proton resonances of the gene-5 protein (Alma et al., 1981a). In the same series of experiments transfer of magnetization was observed between protein and $d(A)_8$ resonances in the protein–$d(A)_8$ complex.

Now that we have assigned the H_2 and the H_8 resonances in this complex (see Figure 2), we can extend the experiments to obtain a more complete picture of the protein–$d(A)_8$ interaction. To this end NOE experiments were performed in which individual H_2 or H_8 proton resonances as well as sugar resonances were specifically preirradiated. Due to the fast exchange conditions, protons of both the free and the complexed $d(A)_8$ molecules are saturated upon preirradiation. For $\omega\tau_c \gtrsim 1$ the rate of cross polarization is roughly proportional to the molecular weight. Hence the observed NOE's are caused predominantly by the $d(A)_8$ molecules to which protein molecules are bound (Clore & Gronenborn, 1982). Different and in many instances quite long preirradiation times were chosen so as to optimize the NOE's, while at the same time keeping nonspecific spin diffusion as low as possible. Even with prolonged irradiation times the intensity of the observed NOE's is about 5–25% of the intensity of the irradiated peak. Examples of these experiments are presented in Figure 5.

Preirradiation of H_8 Protons. In general preirradiation of the H_8 resonances during 1 s gives rise to NOE's for the resonances B/C in the aromatic region of the protein spectrum and at sugar resonance positions, e.g., for the $H_{1'}$ resonances between 5.8 and 6.3 ppm, for the $H_{2'}$ resonances around 2.5 ppm, for the $H_{3'}$ resonances at 5.0 ppm and for the $H_{4'}$ and $H_{5'}$ resonances around 4.2 ppm. An example of such an experiment is presented in Figure 5B. The NOE's observed in this difference spectrum were obtained after 1 s of preirradiation of resonance a5 (for lettering see Figure 2). However, not all of the H_8 resonances give rise to Overhauser effects of the same intensity; e.g., the effects obtained after irradiation of a6 are about the same as those obtained for a5, while preirradiation of peak a8 gives rise to even weaker effects.

Large NOE's are observed after irradiation of a2 and a3. Since these resonances are partly overlapping with resonance M, part of the effects will be the result of saturation of resonance M (see below).

Preirradiation of resonance a1 (the H_8 resonance observed at the lowest field position) gives rise to NOE's presented in Figure 5C. Comparison with Figure 5B reveals that the magnitude of the Overhauser effects is significantly less. Interestingly the effect observed for the $H_{1'}$ sugar resonances is now mainly at 6.2 ppm, the resonance assigned to the 3′-terminal adenyl residue (vide supra). Also the appearance of the NOE for the sugar $H_{2'}$ resonances is different from the NOE's found after irradiation of the other H_8 protons in that an extra resonance is observed downfield from the normal NOE's observed at the $H_{2'}$ resonance positions. This downfield resonance most likely corresponds with the 3′-terminal $H_{2'2}$ sugar proton. This assignment is compatible with the findings of Olsthoorn and collaborators (1980), who observed that the 3′-terminal $H_{2'2}$ sugar proton of triadenylic acid resonates at lower field than the other $H_{2'}$ protons. Therefore resonance a1 most likely belongs to the H_8 protons of the 3′-terminal adenyl ring. This also provides an explanation for the relatively small Overhauser effects: this terminal nucleotide is probably when a protein is bound to the $d(A)_8$ only for a small fraction

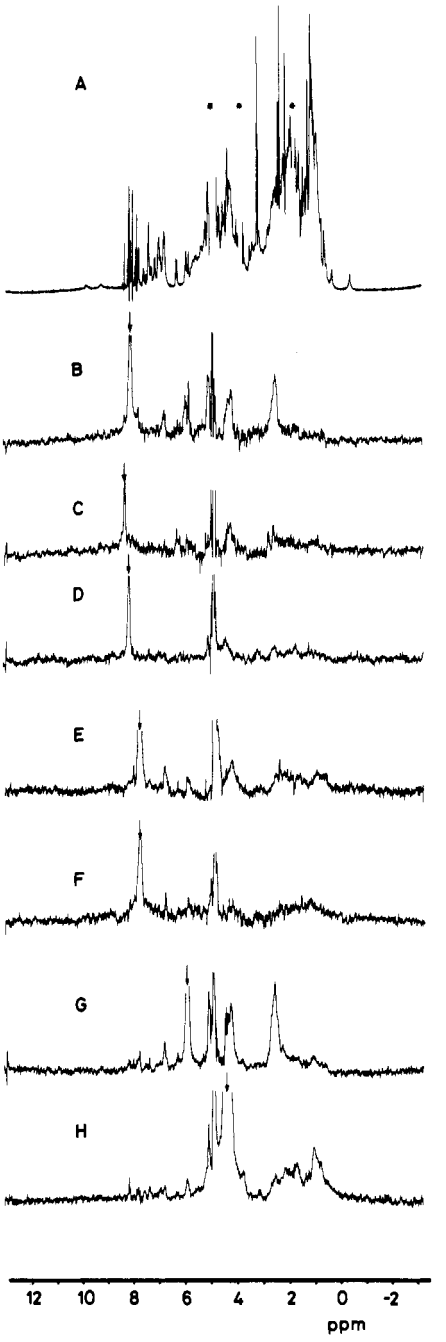

FIGURE 5: NOE difference spectra of the complexes of gene-5 protein with $d(A)_8$ (B, C, F) and H_8-exchange $d(A)_8$ (D, E, G, H). For comparison the 500-MHz ^1H NMR spectrum of the gene-5 protein–$d(A)_8$ complex is given in (A). Peak labels are as given in Figures 2A and 4A. Conditions are as given in Figure 2. The preirradiation frequencies are indicated by arrows. The assignments of peaks a1–a13 are given in Table I. (A) Spectrum of the gene-5 protein–$d(A)_8$ complex. (B) Presaturation of peak a5 during 1.0 s. (C) Presaturation of peak a1 during 1.0 s. (D) Presaturation of peak M during 0.2 s. (E) Presaturation of peak a13 during 4.0 s. (F) Presaturation of peak a13 during 1.0 s. (G) Presaturation of peak a15 (belonging to $H_{1'}$ sugar protons) during 0.3 s. (H) Presaturation of the $H_{4'}$ sugar protons during 0.1 s.

in the direct neighborhood of the protein and therefore relatively mobile (see Discussion).

Preirradiation of Resonance M. Resonance M has been assigned to the C_2 proton of His-64. It overlaps with the H_8 resonances a2 and a3. The relatively large NOE's found after irradiation of resonances a2 and a3 (vide supra) suggest that these NOE's originate (in part) as a result of the simultaneous saturation of resonance M. Therefore the influence of resonance M was studied separately for the complex with H_8-exchanged $d(A)_8$ (compare parts A and B of Figure 2). The effect of a 0.2-s preirradiation pulse on this resonance is shown in Figure 5D. It is clear that NOE's are obtained at the spectral positions of the $H_{3'}$, $H_{4'}/H_{5'}$, and $H_{2'}$ sugar resonances. In addition there are NOE's at positions 3.1 and 1.6 ppm which we cannot assign at the moment. A short preirradiation time was chosen for this experiment to be certain that the observed NOE's were primary effects belonging to protons in the close proximity of the His C_2 proton. This was confirmed by the observation that only the beginning of an NOE is found for the C_4 proton of His (peak 1, visible in an expanded form of Figure 5D) which is one of the protons closest to the C_2 proton within the histidyl residue (distance ~4.5 Å). These results show that His-64 is in or near the DNA binding groove. Interestingly no effects are observed for the $H_{1'}$ protons.

Preirradiation of H_2 Resonances. Preirradiation of the H_2 resonances performed with the complex with H_8-exchanged $d(A)_8$ leads to Overhauser effects in most cases only after a saturation pulse of 4 s in length. An example is given in Figure 5E. Effects are seen for B/C, the $H_{1'}$ sugar resonances, and the $H_{4'}/H_{5'}$ sugar resonances. The effect on the $H_{2'}$ sugar resonances overlaps with NOE's of protein resonances. The sharp H_2 resonances a4 and a6 yield no NOE's even after 4 s of irradiation. Resonances a11 and a13, which yielded the largest NOE after 4-s preirradiation, also gave rise to an NOE in an experiment with 1-s preirradiation and prelonged time averaging (Figure 5F). The specific effect found for peak B/C proves that those H_2 protons are far removed from all other protons in the complex, the closest protons being those resonating in peak B/C.

Preirradiation of Sugar Protons. By irradiation of the adenyl ring protons NOE's were obtained on sugar protons. It is therefore worthwhile to carry out the reverse experiment, i.e., preirradiate the sugar protons. In an earlier paper (Alma et al., 1981a) we have already presented an experiment with the unexchanged $d(A)_8$–gene-5 protein complex in which the sugar $H_{1'}$ protons were preirradiated. In this experiment NOE's were observed for the other sugar protons, for protein resonances A, B/C, and K/I, and for adenyl ring protons resonating at 8.06 and 7.79 ppm. The NOE observed at 8.06 ppm can now be assigned to the H_8 resonances a2 and a3 and probably to resonance M; the NOE observed at 7.79 ppm originates in part from H_8 resonance a8 and for a small part from H_2 resonances. This is shown in Figure 5G, which displays the NOE difference spectrum recorded after 0.3-s preirradiation of the $H_{1'}$ protons in the complex with H_8-exchanged $d(A)_8$. Now the total spectrum is shown, and it is clear that Overhauser effects on the other sugar protons are much higher than those on the adenyl H_2 protons and the protein resonances I, B/C, and A. Therefore these latter NOE's may be secondary effects. In our earlier experiments (Alma et al., 1981a) we were not able to assign the NOE at resonance K/I. Since we now know that the His C_2 proton is close to the sugar $H_{4'}$ proton, it is reasonable to assume that the NOE observed at this position originates from the His C_4 proton.

Irradiation of the other sugar protons may give rise to less straightforward results since they overlap with protein resonances, so that quite easily spin diffusion may occur. For instance, upon preirradiation at the resonance position of $H_{4'}$ and $H_{5'}$ protons also Cα protons are saturated. It can be expected that magnetization from these Cα protons spreads rapidly over the protein. Therefore only those NOE's may be concluded to arise from coupling with $H_{4'}$ (or $H_{5'}$) protons that are already present after short preirradiation times and belong to protons that can be expected to be located far from the Cα protons. This is true for the effects observed for peak M, which is together with the effects at 5.1 and around 5.9 ppm the most prominent feature in the NOE difference spectrum recorded after 0.1 s of preirradiation at the resonance position of the $H_{4'}$ protons (Figure 5H). This observation is in accordance with the experiment shown in Figure 5D. The time dependence of the NOE's is such that the effect for the $H_{2'}$ resonances starts growing after the effect observed for the $H_{3'}$ and $H_{1'}$ resonances, indicating that the effect on the $H_{2'}$ resonances arises (partly) via $H_{3'}$. The specific NOE for resonance M is absent after preirradiation of the $H_{5'}$ resonance.

Preirradiation of Resonance B/C. As observed in Figure 5 irradiation of a resonance of $d(A)_8$ gives rise in many cases to an NOE for the protein resonance B/C. Therefore the reverse experiment, i.e., preirradiation of resonance B/C, was also carried out. A strong spin diffusion is observed in this experiment, and all resonances of $d(A)_8$ seem to give rise to NOE's. Measurement of NOE's as a function of the preirradiation time suggests that these NOE's arise simultaneously; they are present already after 0.1-s preirradiation. This is understandable if we realize that in peak C all aromatic protons of Phe I resonate, which span a width of ~5 Å and can be near different protons of the $d(A)_8$.

NOE Experiments with the Gene-5 Protein–$d(A)_{25-30}$ Complex. In comparison with the gene-5 protein–$d(A)_8$ complex the gene-5 protein–$d(A)_{25-30}$ complex has a much larger molecular weight, and therefore NOE experiments are much more easily complicated by spin-diffusion effects. To be able to observe specific NOE's in spite of the expected strong spin diffusion only short preirradiation pulses can be applied. The optimal pulse length turned out to be 0.05 s, and this was used for all experiments. Examples of NOE difference spectra are presented in Figure 6. Even with the short saturation pulses the occurrence of some spin diffusion could not be prevented; its influence is mainly observed as a broad resonance intensity in the methyl region of the spectrum (see Figure 6). The intensities of the Overhauser effects measured in these experiments are between 2 and 10% of the intensity of the irradiated peak.

Preirradiation of H_8 Adenyl Resonances. Figure 6B presents the NOE difference spectrum recorded after irradiation of resonance aa1 (see Figure 4). At this position the resonances of the adenyl H_8 protons of the free and the complexed $d(A)_{25-30}$ overlap, so that also NOE's are expected for resonances of the free oligonucleotide. This is indeed the case for the $H_{1'}$ resonances of free $d(A)_{25-30}$ at 5.60 ppm (aa5) though its intensity is less than that of the $H_{1'}$ resonance at 5.76 ppm (aa4), which comes from the complexed oligonucleotide. This is to be expected since the transfer of magnetization is faster in the higher molecular weight particle. Other NOE's are observed for the $H_{4'}/H_{5'}$ resonances around 4.1 ppm and for the $H_{2'}$ resonances around 2.3 ppm. Possible effects in the $H_{3'}$ resonances are obscured by the HDO peak. The NOE observed for the B′/C′ resonances is small relative to the corresponding situation in the protein–$d(A)_8$ complex

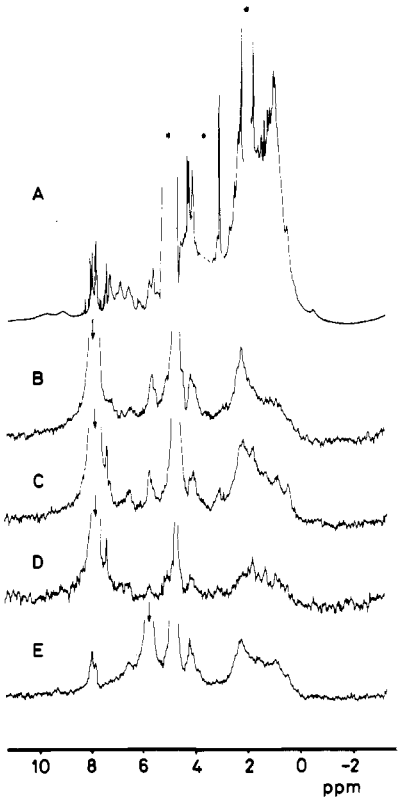

FIGURE 6: NOE difference spectra of the complex of gene-5 protein with excess $d(A)_{25-30}$ (B, C, and E) and with excess H_8-exchanged $d(A)_{25-30}$ (D). Conditions are as given in Figures 3B and 4C. Part A shows the 500-MHz ^1H NMR spectrum of the gene-5 protein–$d(A)_{25-30}$ complex. Peak labels are as shown in Figure 4B. The duration of the preirradiation pulse was 0.05 s in all experiments. The preirradiation frequencies are indicated by arrows. (A) Spectrum of the gene-5 protein–$d(A)_{25-30}$ complex. (B) Presaturation of peak aa1 (resonances of the free and complexed adenyl H_8 protons). (C) Presaturation of peak aa2 (resonances of the complexed adenyl H_2 protons and part of the complexed adenyl H_8 protons). (D) Presaturation of peak aa2 (resonances of the complexed adenyl H_2 protons). (E) Presaturation of peak aa4 (resonance of the complexed sugar $H_{1'}$ protons).

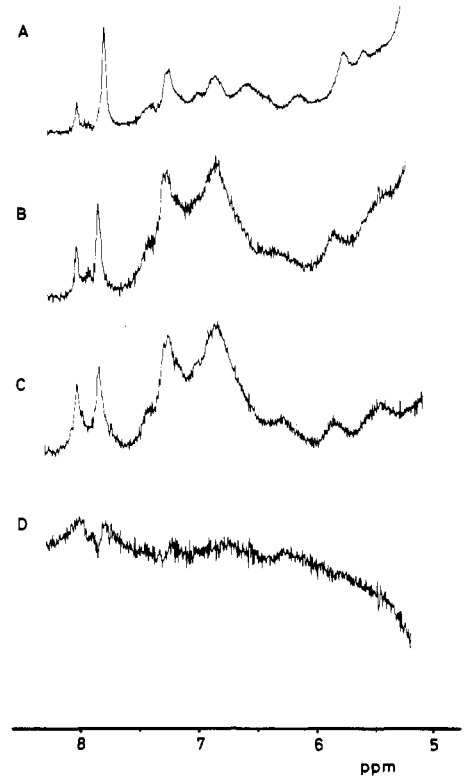

FIGURE 7: Illustration of the changes in the ^1H NMR spectrum between 8.5 and 5 ppm of the gene-5 protein–$d(A)_{25-30}$ complex upon addition of excess protein. (A) 500-MHz ^1H NMR spectrum of the complex of gene-5 protein with excess H_8-exchanged $d(A)_{25-30}$ (same spectrum as shown in Figure 4C). (B) 500-MHz ^1H NMR spectrum of the complex of gene-5 protein with H_8-exchanged $d(A)_{25-30}$ after addition of excess protein. (C) 500-MHz ^1H NMR spectrum of the complex of gene-5 protein with $d(A)_{25-30}$ in the presence of excess protein. (D) Difference spectrum (C) – (B). The shifts of the oligonucleotide resonances are indicated.

(see Figure 5B). Presaturation of the C_2 proton of histidine (resonance M) carried out with the H_8-exchanged $d(A)_{25-30}$–gene-5 protein complex does not give rise to Overhauser effects in contrast to the results found for the gene-5 protein–$d(A)_8$ complex.

Preirradiation of Adenyl H_2 Resonances. Saturation of the adenyl H_2 resonances was carried out with the complex of gene-5 protein with H_8-exchanged $d(A)_{25-30}$. Preirradiation at 7.80 ppm (resonance aa2) yields the NOE difference spectrum presented in Figure 6D. Comparison with Figure 6B shows that apart from some spin-diffusion effects the NOE's are now significantly smaller than after irradiation of the H_8 resonance at 7.9 ppm in accordance with the results obtained for the $d(A)_8$–protein complex. As has been indicated above part of the intensity of the adenyl H_8 resonance is extending beneath the adenyl H_2 resonance. This is clearly seen when the protonated material is irradiated at 7.80 ppm (Figure 6C). Now the observed NOE's are much stronger which we interpret to arise from the saturation of the H_8 resonances beneath the H_2 resonance. In addition the NOE observed for the B′/C′ resonance is much more pronounced than in the experiment represented by Figure 6B. This indicates that the H_8 protons resonating at or close to the H_2 resonance position are in the proximity of Phe I or Tyr IV in the complex. Note that the position of the NOE has shifted with respect to that in the protein–$d(A)_8$ complex just as the B′/C′ resonance has shifted with respect to the B/C resonance (vide supra).

Preirradiation of the Sugar Protons. Preirradiation at the positions of the $H_{2'}$, $H_{4'}$, and $H_{5'}$ resonance positions gives rise to such strong spin-diffusion effects that no structural information can be obtained from the experiments. Saturation of the $H_{1'}$ resonances at 5.76 ppm yields more favorable results. NOE's at the $H_{4'}/H_{5'}$ and $H_{2'}$ positions can now be distinguished (Figure 6E). Downfield from the irradiation frequency the effect on the B′/C′ resonance and on H_8/H_2 resonances is clearly visible. When the same experiment was performed with the complex with H_8-exchanged material a small NOE for the H_2 resonance was observed, in accordance with the reverse experiment (Figure 6D). This means that most of the intensity around 7.8 ppm in Figure 6E is coming from H_8 protons, clearly showing that the H_8 resonances are spread out as a result of complex formation (vide supra).

Preirradiation of Resonance B′/C′. A strong spin diffusion is observed upon preirradiation of peak B′/C′. This prohibits the derivation of structural information from these experiments.

Changes Occurring in the Gene-5 Protein–$d(A)_{25-30}$ Complex upon Addition of Excess Protein. Above it was indicated that in a situation of excess $d(A)_{25-30}$ there is slow exchange of the oligonucleotide between the free and the complexed state. The opposite turns out to be the case when excess protein is present in solution. This is shown in Figure 7. In Figure 7A the spectrum of the protein–$d(A)_{25-30}$ complex is presented.

Only a slight excess of oligonucleotide (H_8-exchanged material) is present (see also Figure 4C). Figure 7B presents a situation in which excess protein is present. If the slow-exchange situation would be maintained upon addition of excess protein, one would expect resonances of the uncomplexed protein to appear in the spectrum and the resonances of the free oligonucleotide to disappear from the spectrum, while the resonances of the complex remain unchanged. This is not observed; the resonances of the free oligonucleotide indeed disappear from the spectrum, but the resonances of the complexed oligonucleotide do not remain at the same spectral positions. Upon addition of gene-5 protein the H_2 and $H_{1'}$ protons shift 0.045 and 0.08 ppm downfield to 7.845 and 5.84 ppm, respectively (compare parts A and B of Figure 7). Addition of more protein causes no further shifts of these resonances. Figure 7C shows the spectrum of the gene-5 protein with unexchanged $d(A)_{25-30}$ in the presence of excess protein. The spectral position of the H_8 resonances can be inferred from the difference spectrum in Figure 7D, which was obtained by subtracting Figure 7B from Figure 7C. Despite the poor signal to noise ratio of this spectrum it can be concluded that the H_8 protons give rise to two broad peaks centered at about 7.8 and 8.0 ppm. This resembles the situation found in the presence of excess oligonucleotide although at that time we could not exactly determine the position of these resonances because they overlap with the resonances of the H_8 of the free oligonucleotide and the H_2 of the bound oligonucleotide (vide supra). The changes in the resonance positions of the H_2 and $H_{1'}$ protons indicate that in the presence of excess protein the structure of the complex formed differs from that in the presence of excess $d(A)_{25-30}$. As to the protein resonances, in the presence of excess protein peaks B′/C′ and (to a lesser extent) peak A shift downfield (for lettering see Figure 4A), while even at a 2-fold excess of protein no resonances of free protein are visible in the spectra; e.g., the Phe I resonances would be expected to appear as sharp peaks around 7.4 ppm (Alma et al., 1981a). This and the form of the aromatic part of the protein spectrum suggest that the protein is in fast or intermediate exchange between the complexed and the free state.

Discussion

Complex of the Gene-5 Protein with $d(A)_8$ in the Presence of Excess $d(A)_8$. The binding characteristics of the gene-5 protein to $d(A)_8$ have been described before (Alma et al., 1981a, 1982). It turned out that, in contrast to the binding of the protein to polynucleotides, approximately three nucleotides were covered by one monomer, when the $d(A)_8$ was saturated with protein or vice versa one $d(A)_8$ molecule binds approximately three protein monomers. Moreover the exchange phenomena connected with the formation and dissociation of the protein $d(A)_8$ complex were rapid on the NMR time scale. This is what we see also in the present experiments. By use of the binding parameters determined in Alma et al. (1982) the fraction of complexed $d(A)_8$ is calculated to be ~0.62 with the amount of oligonucleotide added to the sample. Since the cooperativity parameter characterizing the binding to $d(A)_8$ is ~5, this result means that there will be $d(A)_8$ molecules in solution that are only partly saturated. Theoretical studies by Epstein indicate that in such cases the protein molecules tend to bind in the center of the oligonucleotide (Epstein, 1978).

Since one protein monomer covers three nucleotides, nucleotide protons will be located at three different sites in the DNA binding groove of the protein. In general in each site the nucleotide protons will experience a different magnetic environment giving rise to different shifts of the nucleotide resonances. For the present situation of fast exchange and excess of $d(A)_8$ one would expect that these differences would be averaged out unless there is a preferential binding of the protein to a certain part of the DNA, which is suggested by the results. In particular the relatively large upfield shift of the H_8 proton that is already the most upfield resonating H_8 in the free state suggests that the protein indeed binds preferentially to the center of the oligonucleotide (resonance a8; Table I; Figure 2). Also the differential broadening of the H_2 and H_8 proton resonances in Figure 2 may be interpreted as an indication for preferential binding although exchange effects may also be the cause of the observed broadening. In the presence of preferential binding there will be a number of complexed adenyl residues located adjacent to an uncomplexed adenine. Therefore the H_8 and H_2 protons of these complexed adenines will have resonance positions that deviate from the resonance positions characteristic for the regular complexed state. Also resonances of the adjacent free adenines will have shifted from the resonance position of the free state. For this reason observed resonance positions cannot be interpreted as the weighted average of the resonance positions in the free state and in the complexed state.

For the interpretation of the observed NOE's we also have to take into account the presence of partially saturated $d(A)_8$ molecules. Unoccupied adenyl residues in a partly saturated $d(A)_8$ molecule might contribute to the NOE, and these NOE's could then incorrectly be considered as coming from the complexed state. Fortunately the small magnitude of the NOE observed after irradiation of resonance a1 (see Figure 5C) indicates that the unoccupied residues probably have a large degree of internal mobility and contribute therefore to a minor extent to the observed NOE (resonance a1 belongs to the H_8 proton of the 3′-terminal adenyl residue with is probably only for a small fraction covered by the protein). Below we will return to the discussion of the Overhauser effects.

Cooperativity of Protein Binding to $d(A)_8$ and $d(A)_{25-30}$ Is Different. As indicated above, with excess of $d(A)_8$ molecules present in solution the oligonucleotides will be only partly saturated with protein moleucles. This is in striking contrast to what is observed for protein binding to $d(A)_{25-30}$. When excess of this material is present, signals of the bound and free oligonucleotide are visible separately (Figure 4B). Moreover in the bound state all H_2 protons are in the same magnetic environment even those that give rise to end effects in the free oligonucleotide. These results show that, contrary to the binding to $d(A)_8$, a situation is approached in which the $d(A)_{25-30}$ molecules are either fully covered with protein or totally empty. This indicates that in this case the binding is much more cooperative than for complex formation with $d(A)_8$. In the present experiments we were not able to determine the cooperativity parameter.

Binding of Gene-5 Protein to $d(A)_{25-30}$ in the Presence of Excess $d(A)_{25-30}$ Differs from That in the Presence of Excess Protein. The complexes formed between $d(A)_8$, $d(A)_{12}$, or $d(A)_{16}$ and gene-5 protein are independent of the ratio protein to oligonucleotide at least when proceeding from a very low oligonucleotide concentration up to a 2-fold excess of oligonucleotide (Alma et al., 1982). However, on the basis of the changes in chemical shifts observed for the nucleotide resonances and the observed changes in exchange characteristics when excess protein is added to $d(A)_{25-30}$, we concluded that the complex in the latter situation is different. These observations can be rationalized in view of our results obtained

before (Alma et al., 1982). In the complex of gene-5 protein with $d(A)_8$, $d(A)_{12}$, or $d(A)_{16}$ one protein monomer covers three nucleotide units; on the other hand in a gene-5 protein–polynucleotide complex one protein covers four nucleotide residues (Alberts et al., 1972; Day, 1973; Pretorius et al., 1975; Alma et al., 1983). Therefore it is expected that a transition between the two binding modes will take place when the oligonucleotide exceeds a certain length. The observation of two types of complexes of gene-5 protein with $d(A)_{25-30}$ suggests that we have indeed observed the expected transition. This conclusion is based on the following reasoning. If a protein can bind to a nucleotide chain in two binding modes, theory predicts that the mode in which the smallest number of nucleotide units is covered prevails at high concentrations of free protein (Schwartz & Stankowski, 1979). Given favorable conditions, a switch to a mode in which a higher number of nucleotides is covered can be obtained when one proceeds to low free protein concentrations. We therefore interpret the properties of the complex formed between gene-5 protein and $d(A)_{25-30}$ in the presence of excess oligonucleotide as being characteristic of the gene-5 protein–polynucleotide complex (in the following designated the $n = 4$ binding mode). In the situation of excess protein the complex with $d(A)_{25-30}$ is thought to be similar to the gene-5 protein–$d(A)_8$ complex ($n = 3$ binding mode). This is consistent with the experimentally observed differences between the $d(A)_8$ complex and the $d(A)_{25-30}$ complex formed with excess $d(A)_{25-30}$. In addition, the transition between the two binding modes provides an explanation for the observed changes of the kinetics of the binding process. It can be concluded that the product of the intrinsic binding constant K_{int} and the cooperativity parameter ω is at least an order of magnitude smaller for the $n = 3$ binding mode (Alma et al., 1982) than for the $n = 4$ binding mode (Alma et al., 1983). This explains the observation that the exchange situation changes from a slow into a fast or intermediate exchange situation after formation of the $n = 3$ complex. Intermediate exchange for the $n = 3$ complex is expected on the basis of the observation that $d(A)_{16}$ binding occurs in intermediate exchange (Alma et al., 1982).

Structural Features of Gene-5 Protein–DNA Binding. In a previous paper (Alma et al., 1981a) we have shown by performing NOE experiments that the Tyr I, the Phe I, and/or the Tyr IV residue resides in the DNA binding groove of the protein and takes part in complex formation with DNA by stacking upon adenyl bases. The NOE experiments discussed in the present paper extend these observations. Measurements on H_8 exchanged and normal $d(A)_8$ and $d(A)_{25-30}$ have shown that the H_2 protons of the adenyl bases are far removed from all other protons, protein as well as DNA protons. For most of the H_2 resonances only after 4 s of preirradiation are NOE's for the B/C resonance observed in the gene-5 protein–$d(A)_8$ complex, while for the complex with $d(A)_{25-30}$ only very small effects are seen on peaks B′/C′. In addition, the narrowness of the H_2 resonances in the high molecular weight complex indicates that there are no relaxing protons in the direct surroundings of the H_2 protons. Nevertheless their resonance positions are influenced by the binding of the protein. When the oligonucleotides are saturated with protein [$d(A)_8$ as well as $d(A)_{25-30}$] the H_2 proton resonances shift to a common position although the adenyl residues may be located at three or four different sites in the DNA binding groove of the protein. This shows that it is not the direct influence of residues of the protein but a decrease of the mutual influence of the adenyl bases that causes the observed shifts. Clearly the adenyl bases are destacked in the protein–DNA complex. Presumably no complete destacking is achieved because the H_2 resonance has not reached the position of the H_2 proton of AMP. In contrast the H_8 resonances do not shift to a common position upon complex formation. This can be deduced from the experiments with $d(A)_{25-30}$ (e.g., see Figures 7D and 6E) and is also suggested by the results of Table I for the $d(A)_8$ complex. The main intensity of the H_8 resonances lies around 7.9 ppm while part of it extends to ~7.8 ppm, overlapping with the H_2 resonance. This spread in chemical shift is not unreasonable because some of the adenyl bases are stacked upon the Phe I residue while neighboring bases are stacked upon Tyr I and/or Tyr IV residues in the binding groove. It is interesting to note that in the protein–$d(A)_{25-30}$ complex the H_8 resonances overlapping with the H_2 resonance are fairly the only H_8 resonances giving rise to an NOE on the B′/C′ resonances. We conclude that those H_8 protons are closest to the protons resonating in peaks B′/C′.

There are some differences between the protein–$d(A)_{25-30}$ complex and the protein–$d(A)_8$ complex [which has the same structure as the complexes formed between the protein and $d(A)_{12}$ and $d(A)_{16}$]. One is the shifted resonance position B′/C′ of the $d(A)_{25-30}$ complex with respect to the B/C resonances of the $d(A)_8$ complex. We have argued above that most probably the resonances of Phe I and probably also the 3,5 resonances of Tyr IV are responsible for this shift. Apparently there is a difference in stacking of the Phe I and/or Tyr IV residue upon the adenyl bases in the two complexes. It is also noteworthy that the NOE experiments show that the His-64 C_2 proton is close to the sugar $H_{4'}$ protons in the protein–$d(A)_8$ complex. The His-64 C_4 proton is probably located near a sugar $H_{1'}$ proton. This explains the NOE's observed for peak I after irradiation of the sugar $H_{1'}$ protons in the present and our previous experiments (Alma et al., 1981a). No such effects were observed for the protein–$d(A)_{25-30}$ complex in the presence of excess $d(A)_{25-30}$. In the X-ray structure of the gene-5 protein the histidyl residue is located in the loop that is involved in the dimer interaction (McPherson et al., 1979, 1980a,b), but it seems to be close enough to the presumed binding groove not to be in contradiction with our results.

Possible Structure for Poly(dA) in the Poly(dA)–Gene-5 Protein Complex. To obtain a more pictorial view of the DNA–gene-5 protein complex we propose a structure which conforms to the results discussed above. To this end we will assume that the complex formed between the protein and $d(A)_{25-30}$ with excess oligonucleotide has the same characteristics as that formed between the protein and the poly(dA). Moreover we will need data available from electron microscopy and neutron diffraction measurements. For the helical gene-5 protein–fd DNA complex the following structural features were derived from these studies (Torbet et al., 1981). With a stoichiometry of 4 nucleotides per protein monomer, it is estimated that ~24 nucleotide residues are present per helical pitch of ~90 Å. This is equivalent to a 3.75 Å axial rise and a 15° axial rotation per nucleotide unit. For a phosphate–phosphate distance of 6–7 Å the radial coordinate of the phosphate may have values between 18 and 22 Å. Given an outside diameter of 100 Å for the complex, the main part of the protein must be located outside of the polynucleotide helix.

X-ray data indicate that the basic amino acid residues that are possible candidates for interaction with the phosphates are located primarily in the interior of the DNA binding groove of the protein, while the aromatic residues that may interact with the bases are situated at the exterior of the groove. Hence, the (adenyl) bases are most probably located in the

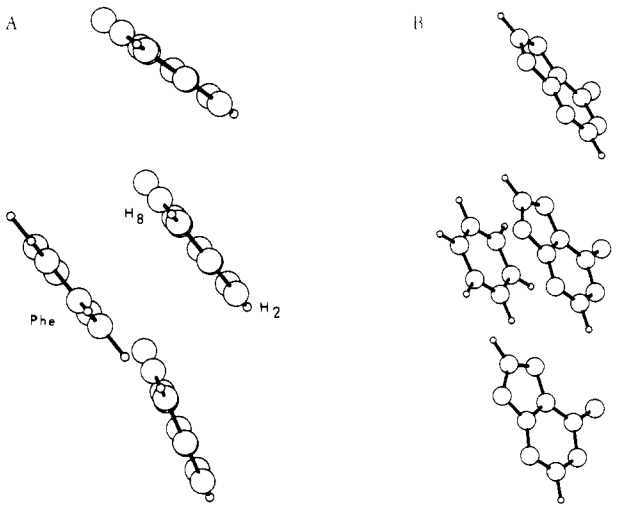

FIGURE 8: Possible conformation of a detail of the gene-5 protein–poly(dA) complex. The drawings show the spatial orientation of three adenyl bases and the phenyl ring of the phenylalanyl residue that is stacked upon the central base. (A) The view is along the helix axis of the complex. The N_9 atom of the central adenine is 19 Å displaced from the helix axis. (B) The complex is viewed along a direction which yields a minimal overlap of the atoms.

interior of the complex, closer to the helical axis than the phosphates.

The above data in conjunction with the data from the NMR experiments described in this paper suggest that the conformation of the sugar phosphate backbone in the complex differs somewhat, though not dramatically, from the conformation in the free state. Yet, the small changes in the sugar phosphate backbone may give rise to a large overall structural alteration of the DNA in the complex. Below we will assume that the helical structure has the features outlined above.

For a further characterization of the nucleotide conformation we will now make use of the resonance positions of the H_2 and H_8 protons in the complex. The H_2 and H_8 resonances have shifted upfield by 0.435 and 0.53–0.66 ppm, respectively, with respect to their positions in pAp (Olsthoorn et al., 1980). These shifts will be caused by the ring currents and anisotropic susceptibilities of neighboring bases and by interactions with the protein for the H_8 resonances. We estimate that the ring current contribution to these shifts is between 0.3 and 0.5 ppm for the H_2 protons and between 0.3 and 0.7 ppm for the H_8 protons. These shifts can be computed with the aid of ring current calculations (see Materials and Methods) for a particular helical structure of the poly(dA). Within the constraints of the helical structure indicated above, a great number of conformations can be generated which give all rise to shifts within these ranges. However, the magnitude of the shifts observed for the protein resonances imposes extra restrictions on the conformation of the poly(dA). With a radial distance of the N_9 atoms of 13–21 Å and the main part of the adenyl bases lying closer to the helix axis, the distance between two adjacent adenyl bases cannot become as large as 6.8 Å in a regular helix. This precludes intercalation of an aromatic protein residue between two bases. Since we found experimentally an average upfield shift of the aromatic resonances of Phe I of ~0.7 ppm upon binding, this residue must stack efficiently upon an adenyl base. On the other hand we have concluded that the position of the adenyl H_2 resonance is virtually not influenced by the protein. Thus, the center of the Phe I aromatic ring must be located relatively far from the H_2 proton. The helical nucleotide conformation must therefore leave enough space for the aromatic protein residues to stack upon the adenyl five-membered ring. The shift of all the aromatic protons of Phe I cannot be generated by a single adenyl residue. Hence there must be an additional adenyl base that contributes to the shift of the Phe protons. These requirements severely restrict the range of possible conformations of the polynucleotide chain. A conformation that conforms to the features outlined above is depicted in Figure 8. Figure 8A shows three of the adenyl bases, with a phenyl ring stacked upon the central base, viewed along the helix axis. In Figure 8B the same part of the complex is drawn, now seen from a direction in which the overlap of atoms is at a minimum. It is clear from these drawings that in this particular conformation the planes of the adenyl bases are parallel to the helix axis. The phenylalanyl ring is assumed to be parallel to the central adenyl base. A slight tilt of the Phe ring might be needed to diminish the ring current shift that the Phe ring exerts on the H_2 proton of the uppermost adenyl base. The H_8 proton belonging to central adenine is expected to undergo an upfield shift. In the proposed conformation the glycosidic torsion angle (χ_{CN}) is about 0°, and the sugar proton that is closest to the Phe is the $H_{1'}$ proton.

Although the proposed conformation is tentative, it is consistent with the data available to date, and it explains why the bases cannot be involved in base pairing.

Conclusions

In this paper we have demonstrated that, in the presence of excess oligonucleotide, the gene-5 protein forms a complex with $d(A)_{25-30}$ which is different from the complex with smaller oligonucleotides. The differences are manifested both by the shift of the aromatic resonances of Phe I in the ^1H NMR spectrum of the complexed protein and by the cooperativity and kinetics of the complex formation.

The complex of the gene-5 protein with $d(A)_{25-30}$ undergoes a structural transition when proceeding from excess oligonucleotide to excess protein. This transition has been identified as the transition between the polynucleotide and the oligonucleotide binding mode. The aromatic protein residues Phe I, Tyr I, and probably Tyr IV had been shown earlier to be involved in DNA binding. The present NOE experiments have shown that also the single histidyl residue is located near the oligonucleotide in the gene-5 protein–$d(A)_8$ complex. The orientation of the oligonucleotides in the complex is such that the adenyl H_8 and sugar $H_{1'}$ protons are situated near the protein, while the adenyl H_2 protons are relatively far removed from all other protons. By combining the structural information which is presently available we have been able to propose a possible orientation of the adenyl bases in the complex.

Acknowledgments

We thank Dr. C. A. G. Haasnoot and ing. P. A. W. van Dael for keeping the NMR spectrometer in excellent condition. Professor Dr. J. G. G. Schoemakers and Dr. R. H. N. Konings are acknowledged for stimulating discussions, and Carla Prinse is acknowledged for technical assistance in the preparation of the gene-5 protein and N. Kersten-Piepenbrock for the preparation of the manuscript.

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