^{13}C NMR of Methylated Lysines of fd Gene 5 Protein: Evidence for a Conformational Change Involving Lysine 24 upon Binding of a Negatively Charged Lanthanide Chelate†

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ABSTRACT: Helical complexes formed between fd DNA and reductively methylated fd gene 5 protein were indistinguishable by electron microscopy from complexes formed with the nonmethylated protein. ^{13}C NMR spectroscopy of ^{13}C-enriched N^{ϵ},N^{ϵ}-dimethyllysyl residues of the protein showed that three of these residues (Lys-24, Lys-46, and Lys-69) were selectively perturbed by binding of the oligomer $d(pA)_7$. These were the same lysyl residues that we previously found to be most protected from methylation by binding of the protein to poly[r(U)] [Dick, L. R., Sherry, A. D., Newkirk, M. M., & Gray D. M. (1988) *J. Biol. Chem. 263*, 18864-18872]. Thus, these lysines are probably directly involved in the nucleic acid binding function of the protein. Negatively charged chelates of lanthanide ions were used to perturb the ^{13}C NMR resonances of labeled lysyl and amino-terminal residues of the gene 5 protein. The terbium chelate was found to bind tightly ($K_a \approx 10^5$ M^{-1}) to the protein with a stoichiometry of 1 chelate molecule per protein dimer. ^{13}C resonances of Lys-24, Lys-46, and Lys-69 were maximally shifted by the terbium chelate and were maximally relaxed by the gadolinium chelate. Also, the terbium chelate was excluded by the oligomer $d(pA)_7$. Computer fits of the induced chemical shifts of ^{13}C resonances with those expected for various positions of the terbium chelate failed to yield a possible chelate binding site unless the chemical shift for Lys-24 was excluded from the fitting process. With this exclusion, a possible chelate binding site was found to be a hydrophobic pocket centered between the two DNA-binding loops of the protein dimer. Chelate-induced shifts that could be measured for ^{1}H resonances agreed with this location of the binding site. All of our data would be consistent with this location if there were a substantial movement of the flexible DNA-binding loops containing Lys-24 upon binding of the chelate.

The bacteriophage fd gene 5 protein (G5P)[1] binds strongly and cooperatively to single-stranded nucleic acids. A major contribution to the energy for binding of gene 5 protein to polynucleotides is from electrostatic interactions, which cause neutralization of three to four charges upon binding of a protein monomer to four nucleotides of DNA (Porschke & Rauh, 1983; Alma et al., 1983a). These electrostatic interactions probably involve ion pairing of positively charged lysine and arginine protein side chains with the negatively charged DNA phosphates. A Raman scattering study has provided direct evidence for such interactions (Otto et al., 1987). Evidence for the general involvement of the lysyl residue side chains in the binding of gene 5 protein to DNA has been obtained from chemical modification data (Anderson et al., 1975) as well as from proton NMR spectroscopy (Alma et al., 1981), but neither of these studies implicated specific lysyl residues.

In previous work, we showed that three of the six lysyl residues of the fd gene 5 protein, lysines 24, 46, and 69, are significantly protected from a chemical modification reaction (reductive methylation) when the protein is bound to a polynucleotide, suggesting that these three residues form part of the nucleic acid binding site (Dick et al., 1988). The methylation reaction was also used to label the lysyl residues with ^{13}C-enriched methyl groups, and a partial assignment of the ^{13}C resonances to specific protein lysyl residues was obtained. A structural model of the DNA-binding site that was derived from X-ray crystallography of the gene 5 protein and from modeling studies (Brayer & McPherson, 1983, 1984) only includes Lys-46 in the DNA–protein interactions, although the possible involvement of Lys-24 and Lys-69 was not excluded.

In the present work, ^{13}C NMR spectroscopy was used to monitor perturbations of the modified lysyl residues of the protein upon binding of a DNA oligomer, $d(pA)_7$. Spectral changes caused by the axially symmetric NMR shift probe $Tb(DOTP)^{5-}$ (Sherry et al., 1987) and by the corresponding relaxation probe $Gd(DOTP)^{5-}$ were also measured. The negatively charged $Tb(DOTP)^{5-}$ was excluded from binding to the protein by the DNA oligonucleotide, as shown by a

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[1] Abbreviations: G5P, gene 5 protein; $La(DOTP)^{5-}$, $Tb(DOTP)^{5-}$, and $Gd(DOTP)^{5-}$, the lanthanum(III), terbium(III), and gadolinium(III) chelates, respectively, of 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetrakis(methylenephosphonic acid); LIS, lanthanide-induced chemical shift.

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competition experiment. We were able to show that the resonances corresponding to Lys-24, Lys-46, and Lys-69 were perturbed by binding of the DNA oligomer and that these perturbations were different from those observed upon binding of simple counterions (NaCl). It thus seems likely that these three lysyl residues do directly participate in the protein's nucleic acid binding function.

Various transition-metal and lanthanide ion complexes have previously been used as NMR probes to study their interaction with surfaces of proteins (Eley et al., 1982; Williams et al., 1984). However, this is the first work of which we are aware in which such probes have been used to study a DNA-binding protein. Using the distance and orientation dependence of the effects of these probes on the ^{13}C NMR of the methylated lysyl groups, we located the site on the protein where the probes probably bind. The data indicated that the DNA-binding arm containing Lys-24 can undergo a substantial change in position from its crystallographic coordinates.

In this work we also used electron microscopy to show that the methylated fd gene 5 protein forms structurally normal complexes with single-stranded fd DNA.

Materials and Methods

Protein Isolation and Chemical Modification. The fd gene 5 protein was isolated by the method of Day (1973) with minor modifications. Fractions containing gene 5 protein from a DNA–cellulose column were concentrated by ultrafiltration and then chromatographed on a Sephadex G-75 column to remove high molecular weight contaminants (Anderson et al., 1975). Protein concentrations were calculated from absorption spectra of the protein by using a molar extinction coefficient of 7070 $M^{-1}\cdot cm^{-1}$ at 276 nm (Day, 1973).

Reductive methylation of the protein was carried out by using the method of Jentoft and Dearborn (1983) to modify the lysyl residue ϵ-amino groups with [^{13}C]formaldehyde (KOR Isotopes). As in previous work (Dick et al., 1988), conditions were such that all six lysyl residues of the protein were dimethylated to form the ^{13}C-enriched $N^{\epsilon},N^{\epsilon}$-dimethylysyl derivative of gene 5 protein, while the amino terminus of the protein was only slightly methylated.

Oligodeoxynucleotide. The $d(pA)_7$ (lot no. 0063783) was purchased from Pharmacia. Approximately 25 A_{260} units of lyophilized material was resuspended in 330 μL of the buffer used for the protein samples (see below). The concentration of a dilution of the stock was determined from the UV absorption spectrum by using an extinction coefficient of 10040 $M^{-1}\cdot cm^{-1}$ at 260 nm (Kansy et al., 1986).

1,4,7,10-Tetraazacyclododecane-N,N',N'',N'''-tetrakis(methylenephosphonic acid) (DOTP). Synthesis and characterization of DOTP has been described previously (Sherry et al., 1988). The terbium(III) chelate, $Tb(DOTP)^{5-}$, was prepared by mixing a 20 mM solution of DOTP (free acid in H_2O) with an aliquot of a 117 mM solution of $TbCl_3$ to give a final ratio of 1.00:0.95 DOTP:Tb. The mixture was brought to a pH of 8.7 by titrating it with a solution of 1 M Tris base. Water was then added to yield a solution containing 10 mM $Tb(DOTP)^{5-}$ and 0.29 M Tris-HCl, pH 8.7. $Gd(DOTP)^{5-}$ and $La(DOTP)^{5-}$ were prepared similarly. For the ^1H NMR measurements, NH_4OH was used to adjust the pH of $Tb(DOTP)^{5-}$ to 9.

NMR Spectra. Methylated gene 5 protein samples were concentrated to 3–5 mg/mL and dialyzed into the desired buffer. The buffer used for the ^{13}C NMR experiments contained 10 mM NaCl and 10 mM Tris-HCl, pH 9.0 at 25 °C, with 1% (v/v) methanol to serve as an internal ^{13}C chemical shift standard at 49.4 ppm. A field-frequency lock was provided by adding D_2O to a final concentration of 10% (v/v). Addition of $Tb(DOTP)^{5-}$ or $La(DOTP)^{5-}$ entailed a small ($\approx$10%) additional dilution of the protein with a concomitant increase in the concentration of Tris ($\approx$2-fold) and a slight lowering of the sample pH (9.0 to 8.7). Addition of $Gd(DOTP)^{5-}$ did not appreciably change any of the solution variables. Changes in these variables ([protein], [Tris], and pH) within the ranges encountered for the experiments had no significant effect on the appearance of the ^{13}C NMR spectra. ^1H NMR spectra were taken with the protein in D_2O containing 20 mM Na^+ (phosphate), pD 9.5. Protein concentrations were determined from absorption spectra, as described above, and are indicated in the figure legends.

Spectra were taken at 50.1 MHz on a JEOL FX-200 FT NMR with broad-band proton decoupling (WALTZ decoupling). The temperature of the samples was 28 $\pm$ 2 °C at the decoupling power level used. Typically, 1000 scans using a 60° pulse and a 2.5-s delay between pulses were sufficient to achieve a reasonable signal-to-noise ratio. Nuclear spin–lattice relaxation times were measured by using an inversion–recovery pulse sequence. Some ^{13}C NMR spectra and spin–lattice relaxation time measurements were also carried out at 125.7 MHz on a General Electric GN-500 FT NMR with WALTZ proton decoupling. The ^1H NMR spectra were recorded at 500.14 MHz on the latter instrument.

Computer Analysis of the Chelate Binding Position. A computer program (Sherry & Teherani, 1983) was used to calculate LIS values for the ^{13}C NMR resonances of the dimethylated lysyl and amino-terminal residues as a function of $Tb(DOTP)^{5-}$ position within a protein dimer. This program allowed movement of the lanthanide·DOTP chelate over the surface of spheres centered at the origin of the crystallographic structure of the protein. For each position of the $Tb(DOTP)^{5-}$, the principal symmetry axis of the chelate was also allowed to vary. LIS values were calculated for each lysyl and amino-terminal residue in the ^{13}C NMR spectrum and for several other residues identified in the ^1H NMR spectrum; these values varied with a $(3\cos^2\theta - 1)/r^3$ dependence on the distance (r) between the chelate and the amino acid residue and on the angle (θ) between the principal symmetry axis of the chelate and the vector from the chelate to the residue. LIS values were thus calculated for all reasonable chelate positions that contacted the protein dimer, and the calculated values were compared with those obtained experimentally. Coordinates of the amino acid residues in the crystallographic structure of the fd gene 5 protein (Brayer & McPherson, 1983) were provided by A. McPherson (University of California, Riverside).

For the purpose of visualizing the $Ln(DOTP)^{5-}$·G5P complex, the gene 5 protein structure was combined with a molecular model of the chelate and was displayed and photographed at the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center at Dallas) on an Evans and Sutherland PS 300 Graphics System with the Biosym Insight molecular modeling program.

Electron Microscopy. Complexes were formed by titrating single-stranded fd viral DNA with nonmethylated or ^{13}C-methylated fd gene 5 protein in 5 mM Tris-HCl (pH 7.1) or 10 mM ammonium acetate (pH 6.9) to a ratio of 1 protein monomer per 4 DNA nucleotides. The complexes were then diluted to 0.1 mg/mL in 10 mM ammonium acetate (pH 6.9). Glutaraldehyde cross-linking was not used to stabilize the complexes, because the [^{13}C]methyl groups in the methylated protein block the amines that are required for reaction with the bifunctional glutaraldehyde reagent. A film of carbon was

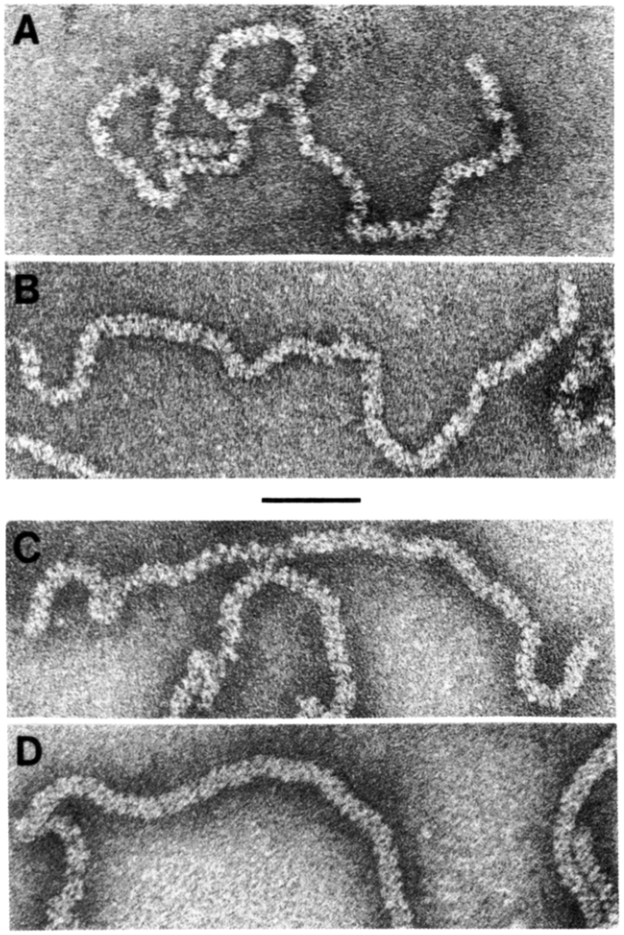

FIGURE 1: Electron micrographs of complexes formed by titrating single-stranded fd viral DNA with ^{13}C-methylated fd gene 5 protein (A, B) or with nonmethylated protein (C, D). The complexes are helices; the complex in panel A has a short Y-branch, giving the false appearance of a loop. Bar = 50 nm.

floated from mica onto the surfaces of the solutions containing the complexes; the complexes became adsorbed to the freshly exposed undersurface of the carbon film [method of Valentine et al. (1968)]. After 30 s, the carbon film was rinsed by transferring it to one or more solutions of 10 mM ammonium acetate (pH 6.9), and it was then transferred to a 2% aqueous uranyl acetate stain solution from which the film was picked up on a copper mesh grid. Kodak 4489 electron image film was exposed in a Carl Zeiss EM10CA electron microscope at an electron-optical magnification of 53000×, using minimal-dose focusing techniques after correcting the instrument astigmatism at 200000× on the background grain in a lightly stained area distant from the specimens. Magnifications were calibrated to ±3% (combined errors) by using a Fullam diffraction grating replica (2160 lines/mm ± 0.3%).

Results

Electron Microscopy. An important question is, to what extent does methylation of the lysines of the fd gene 5 protein affect the structure or DNA-binding properties of the protein? One approach to answering this question is to determine whether the methylated fd gene 5 protein forms structurally normal complexes with single-stranded DNA. Previous electron microscopic studies have shown that the fd gene 5 protein forms a characteristic nucleoprotein helix with DNA single strands (Alberts et al., 1972; Gray et al., 1982). Figure 1 shows a comparison of electron micrographs of complexes formed by titrating fd viral DNA with methylated versus nonmethylated fd gene 5 proteins. As is seen by comparing

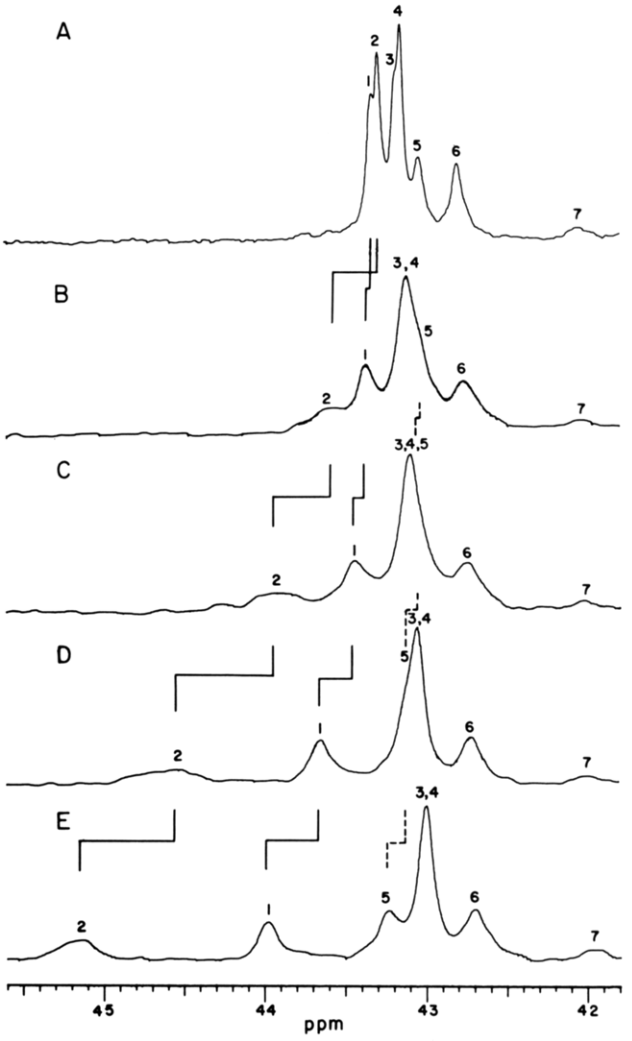

FIGURE 2: 50.1-MHz ^{13}C NMR spectra of ^{13}C-methylated fd gene 5 protein at increasing Tb(DOTP)$^{5-}$ concentrations to give [chelate]/[protein monomer] molar ratios of 0 (A), 0.1 (B), 0.2 (C), 0.4 (D), and 1.0 (E). The protein monomer concentration was 0.35 mM at the start of the titration.

panels A and B (methylated) with panels C and D (nonmethylated), the same helical structure was observed in both cases. The helical pitch ranged from 5 to 7 nm, and the measured widths of the helices varied from 9 to 11.5 nm, for complexes formed with either methylated or nonmethylated protein. A variable pitch is a characteristic property of these flexible nucleoprotein coils (Gray et al., 1982); accordingly, some of the helical turns in panels A and C of Figure 1 are well separated, whereas most of the helical turns in panels B and D are in closer juxtaposition. The nonconstant widths occurred due to partial flattening of the helices in many regions as they adsorbed to the carbon substrate. (The methylated helix in Figure 1A is least flattened, and it has the same width as do segments of the nonmethylated helix in Figure 1C.) In summary, we found that the methylated and nonmethylated complexes were indistinguishable in their overall structure.

Chemical Shifts Induced by Binding of Tb(DOTP)$^{5-}$ to G5P. Figure 2A shows the 50.1-MHz ^{13}C NMR spectrum of the ^{13}C-methylated G5P at pH 8.8, where resonances corresponding to each of the six protein lysyl residues were resolved, numbered 1–6 from high to low frequency. These resonances have been partially assigned to specific lysyl residues from previous work (Dick et al., 1988). Resonance 4 definitely corresponds to Lys-87. Either resonance 1 or resonance 2 corresponds to Lys-24, with the other resonance

Table I: Comparison of Experimental with Calculated Dipolar Shifts Induced by Tb(DOTP)$^{5-}$ for ^{13}C NMR Resonances of ^{13}C-Methylated G5P and Several ^{1}H NMR Resonances of Nonmethylated G5P

		solution 1			solution 2		
resonance[e]	exptl[a] LIS (ppm)	residue	calcd LIS (ppm)	distance[b] (Å)	residue	calcd LIS (ppm)	distance[b] (Å)
			^{13}C NMR Spectra				
1	0.91	Lys-69	0.90	11.5	Lys-46	0.92	13.2
2	2.38	Lys-24	(−0.05)[c]	21.9	Lys-24	(−0.28)[c]	21.4
3	−0.03	Lys-7	−0.07	20.9	Lys-7	−0.07	22.4
4	−0.10	Lys-87	−0.08	23.0	Lys-87	−0.08	24.5
5	0.23	Lys-46	0.26	14.1	Lys-69	0.20	13.5
6	−0.10	Lys-3	−0.09	22.1	Lys-3	−0.09	23.8
7	−0.05	Met-1	−0.06	26.1	Met-1	−0.05	27.7
			^{1}H NMR Spectra				
−0.47	−0.11[d]	Leu-83	−0.10	18.5	Leu-83	−0.20	19.9
2.23	−0.06[d]	Met-1 or Met-77	−0.07 −0.52	24.1 9.9	Met-1 or Met-77	−0.06 −0.25	25.9 13.1
6.51		Tyr-26	−0.07	24.3	Tyr-26	−0.07	25.0
7.38		Phe-73	0.16	14.0	Phe-73	0.23	15.3
8.07	−0.03[d]	His-64	−0.21	15.9	His-64	−0.12	18.4

[a] Positive LIS values represent paramagnetic shifts to higher frequency (lower field). [b] Distances are from the calculated Tb(DOTP)$^{5-}$ binding site to the crystallographic coordinates of the N or C atoms that would be bonded to the resonant ^{13}C or ^{1}H nuclei, respectively. [c] Calculated LIS values for Lys-24 with this residue in its crystallographic position. [d] Extrapolated LIS values based on data obtained from three to four additions of Tb(DOTP)$^{5-}$ to nonmethylated G5P. [e] For ^{13}C NMR spectra, resonance numbers are given. For ^{1}H NMR spectra, resonance positions in ppm are given.

belonging to Lys-46 or Lys-69; resonance 5 is also assigned to either Lys-46 or Lys-69. Finally, resonances 3 and 6 are related, in some order, to Lys-3 and Lys-7. The resonance numbered 7, of lower intensity, corresponds to the α-amino group of the N-terminal Met-1 residue, which is less reactive in the dimethylation reaction (Dick et al., 1988).

Figure 2B–E shows the ^{13}C spectrum of the ^{13}C-methylated G5P in the presence of increasing amounts of Tb(DOTP)$^{5-}$. The gradual increase in the magnitudes of the paramagnetic shifts induced by the binding of Tb(DOTP)$^{5-}$ indicated that fast-exchange conditions applied at this frequency. (This was not the case at 125.7 MHz, where resonance 2 was broadened by exchange so that it was nearly undetectable; data not shown). Resonances 1, 2, and 5 shifted to higher frequencies, and resonances 3, 4, 6, and 7 shifted to slightly lower frequencies during the course of the titration.

Figure 3 shows the chemical shift of each resonance as a function of the chelate/protein ratio for a more complete titration set. The binding of Tb(DOTP)$^{5-}$ to G5P was saturable, and the maximum chemical shift was reached at close to a binding ratio of about 0.5 [chelate]/[protein monomer] (from the data for resonance 2, which showed the maximum shift). These data indicated that there was a single high-affinity binding site (with an association constant $K_a \approx 10^5$ M^{-1}) for the chelate on each protein dimer. Lanthanide-induced chemical shift (LIS) values were obtained from the data by subtracting the ^{13}C shifts of a 1:1 mixture of ^{13}C-methylated G5P/La(DOTP)$^{5-}$ as the diamagnetic reference (see Table I, column 2). The largest LIS values were observed for resonances 1, 2, and 5, which from previous work correspond in some order to Lys-69, Lys-24, and Lys-46. Thus, in addition to their proposed involvement in the DNA-binding site (Dick et al., 1988), these three residues were apparently nearest the chelate binding site.

The 500.14-MHz ^{1}H NMR spectrum of nonmethylated G5P was also examined in the presence and absence of Tb(DOTP)$^{5-}$ (data not shown). Resonances assigned to Phe-73 (3,4,5)H and Tyr-26 (3,5)H (King & Coleman, 1987) completely disappear from the spectrum upon the addition of only 20% of the [Tb(DOTP]$^{5-}$ needed to fill all of the available binding sites. This indicated that these two resonances had large chemical shifts in the presence of the paramagnetic

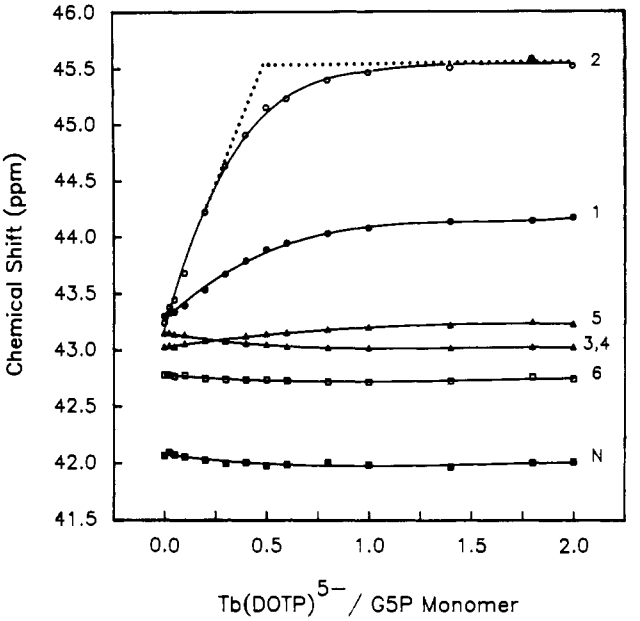

FIGURE 3: Induced chemical shifts as a function of the [chelate]/[protein monomer] ratio for the ^{13}C resonances of ^{13}C-methylated fd gene 5 protein in the presence of Tb(DOTP)$^{5-}$. The chemical shifts for a sample with a [chelate]/[protein monomer] ratio of 4 were the same as the maximal values shown in the figure. The dotted lines illustrate that the binding ratio obtained from the data for resonance 2 (which shows the maximum chemical shift) was about 0.5.

chelate and that the complexed protein was in intermediate exchange with the free protein. Other previously assigned resonances, including the C2H resonance of His-64 near 8.03 ppm and one of the methyl resonances of Leu-83 near −0.52 ppm (King & Coleman, 1987), do shift gradually upon titration with Tb(DOTP)$^{5-}$, but they broaden beyond detection after a [chelate]/[protein monomer] ratio of about 0.3 is reached. A single methionine methyl resonance at 2.23 ppm (from Met-1 or Met-77) also shifts upfield and broadens beyond detection upon addition of Tb(DOTP)$^{5-}$. Maximum LIS values were estimated from these chemical shifts by extrapolating to a [chelate]/[protein monomer] ratio of 0.5, the stoichiometry observed for the ^{13}C resonances of the methylated protein (Figure 2), and assuming that the effects of a

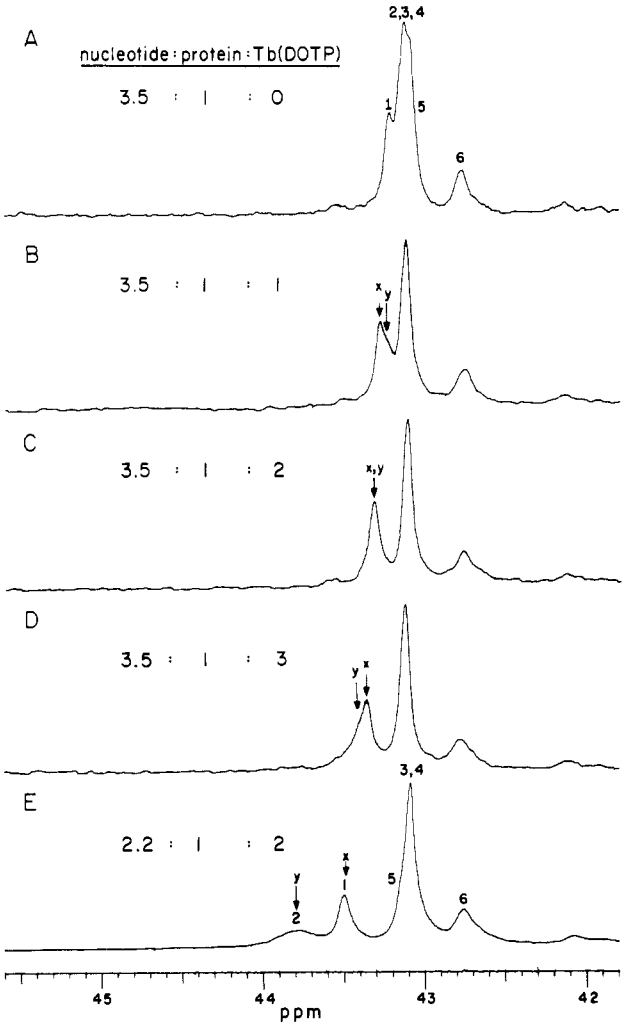

FIGURE 4: 50.1-MHz ^{13}C NMR spectra of the gene 5 protein–$d(pA)_7$ complex with $Tb(DOTP)^{5-}$ at [chelate]/[protein monomer] molar ratios of 0.0 (A), 1.0 (B), 2.0 (C), and 3.0 (D). Panel E is the spectrum of a sample after the addition of more protein, with the relative ratios of components indicated. Protein monomer concentrations were 0.52, 0.50, 0.47, 0.45, and 0.51 mM for the spectra in panels A, B, C, D, and E, respectively.

diamagnetic reference would be small. Although these ^1H LIS values were considerably less accurate than the ^{13}C LIS values, they helped to validate the lanthanide–chelate binding position determined from the ^{13}C LIS values (see below). All of the experimental LIS values are listed in column 2 of Table I.

Competitive Binding of $Tb(DOTP)^{5-}$ and $d(pA)_7$. Figure 4 shows spectra of ^{13}C-methylated G5P in the presence of differing amounts of $d(pA)_7$ and $Tb(DOTP)^{5-}$. Panels A–D show spectra at increasing concentrations of $Tb(DOTP)^{5-}$, with close to saturating concentrations of $d(pA)_7$ (corresponding to at least 0.88 equiv of DNA per binding site, assuming that the binding site of a protein monomer covers no more than four nucleotides; Kansy et al., 1986). In panel B, two resonances, marked x and y, were only slightly shifted to higher frequency relative to their original position (which is shown in panel A), although the concentration of the chelate was beyond that needed to obtain the full chemical shift in the absence of oligonucleotide (Figure 3). As the concentration of the chelate was raised to give a [chelate]/[protein monomer] ratio of 3, resonances x and y shifted further to higher frequency and exchanged their relative positions.

Figure 4E shows the effect of increasing the protein concentration so that there was an excess of protein over the oligonucleotide (0.55 equiv of DNA per protein binding site), but still with an excess of the chelate over the saturating [chelate]/[protein monomer] ratio in the absence of oligonucleotide. The gradual nature of the shifts seen in Figure 4 suggested that both the chelate and the oligonucleotide binding to G5P were in fast exchange on the NMR time scale. The chemical shifts of the resonances in Figure 4 were quite small, relative to those observed at saturating concentrations of the chelate in the absence of oligonucleotide (Figures 2 and 3). This indicated that the binding of the chelate and oligonucleotide were mutually exclusive, the chelate being a poor competitor of the oligonucleotide. If this was indeed the case, the fractional chemical shifts observed in Figure 4 should represent the fraction of chelate-bound protein, and the ratio of the chemical shifts for any two resonances should be the same in the presence or absence of the oligonucleotide. The chemical shift ratios remained constant only if resonance 2 was assumed to be shifted to lower frequency (higher field) relative to resonance 1 upon binding of the oligonucleotide in the absence of $Tb(DOTP)^{5-}$. Thus, resonances 1 and 2 were those marked by x and y, respectively, in Figure 4, and the ^{13}C NMR spectrum of the G5P·$d(pA)_7$ complex could be interpreted as shown in Figure 4A.

Binding of the DNA Oligomer $d(pA)_7$ to ^{13}C-Methylated G5P. Changes in the spectrum caused by oligonucleotide binding (Figure 4A) mimicked to some extent the effects of added salt [see Figure 4C in Dick et al. (1988)]. There were exceptions, however. Upon binding of $d(pA)_7$, resonance 5 shifted to higher frequency and became obscured by the envelope of resonances 1–4, all of which showed shifts of varying degrees to lower frequency. In contrast, the addition of salt causes resonance 5 to shift to lower frequency. Thus, the perturbation of resonance 5 (Lys-46 or Lys-69) observed in Figure 4A was a specific effect of nucleotide binding.

Resonances 1 and 2 (one of which was Lys-24 and the other either Lys-46 or Lys-69) also showed shifts upon oligonucleotide binding, both to lower frequency. Both shifts were larger than the corresponding salt effects.

T_1 Relaxation Values of Dimethylated Lysines 24, 46, and 69 Are Selectively Affected by Lanthanide·DOTP Chelates. The ^{13}C spin–lattice relaxation (T_1) times were measured for the ^{13}C-enriched dimethylated residues of G5P in the absence and the presence of various lanthanide·DOTP chelates. The effect of the diamagnetic probe $La(DOTP)^{5-}$ on the T_1 values, measured both at 50.1 MHz and at 125.7 MHz, was such that, within experimental error, only resonances 1, 2, and 5 were affected. These three T_1 values were reduced (from 0.80 to 0.70, from 0.97 to 0.81, and from 0.70 to 0.60 s, respectively), indicating that the binding of $La(DOTP)^{5-}$ to G5P caused some degree of immobilization of those lysyl residues. The T_1 values of all residues were independent of frequency.

We also measured the T_1 values of the resonances in the presence of paramagnetic lanthanide·DOTP chelates, $Tb(DOTP)^{5-}$ at 50.1 MHz, and $Gd(DOTP)^{5-}$ at 125.7 MHz. Only the T_1 values of resonances 1 and 5 were significantly reduced (from 0.70 to 0.52 and from 0.60 to 0.53 s, respectively) by the paramagnetic ion chelates, relative to the effects of the diamagnetic ion chelate, $La(DOTP)^{5-}$. However, as mentioned above, resonance 2 was broadened so that it could not be detected in the $Tb(DOTP)^{5-}$·G5P complex at 125.7 MHz; this indicated that there was an exchange contribution to the line width (and T_1) of this resonance, which could affect the spectrum of the $Tb(DOTP)^{5-}$·G5P complex at 50.1 MHz. Thus, the measured relaxation value for resonance 2 at 50.1 MHz was only an upper limit, and the relaxation time of this resonance was not related in a simple fashion to the distance

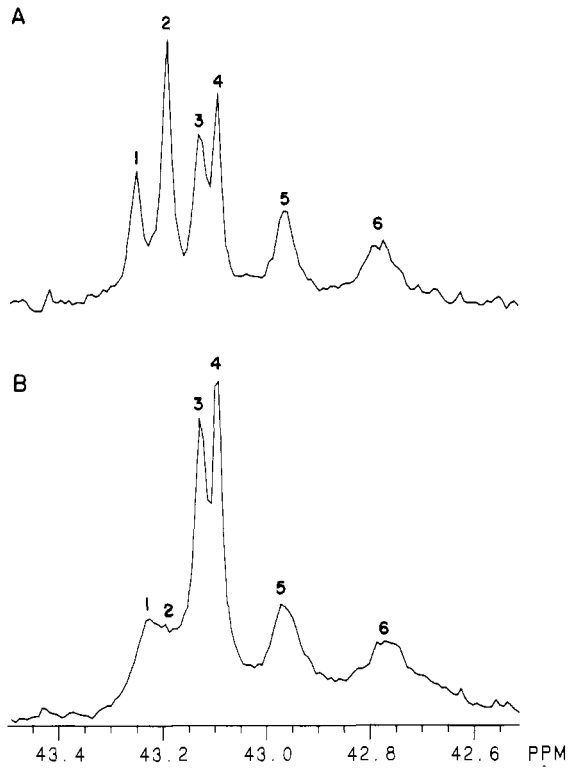

FIGURE 5: 125.7-MHz ^{13}C NMR spectra of ^{13}C-methylated fd gene 5 protein at 0.35 mM concentration in the absence (A) and the presence (B) of 1.95×10^{-5} M Gd(DOTP)$^{5-}$.

between the lanthanide ion and the lysyl residue corresponding to resonance 2, as confirmed by an experiment with Gd(DOTP)$^{5-}$, described next.

The relative distances between the lysines corresponding to resonances 1, 2, and 5 and the chelate binding site were clarified by the use of Gd(DOTP)$^{5-}$, which induces no chemical shift and for which the enhancement of the relaxation rate is solely distance-dependent (r^{-6}). Figure 5 shows a comparison of a spectrum of ^{13}C-methylated G5P at 125.7 MHz in the absence and presence of a very small amount (1.95×10^{-5} M) of Gd(DOTP)$^{5-}$. The paramagnetic line-broadening effect (i.e., the decrease in T_2) due to Gd(DOTP)$^{5-}$ clearly affected the resonances in the following order: resonance 2 $\gg$ resonance 1 $>$ resonance 5. Therefore, we inferred that, qualitatively, the lysyl residue corresponding to resonance 2 must be closer to the Gd(DOTP)$^{5-}$ binding site than were the other labeled lysines. Also, the lysyl residue related to resonance 1 was closer to the chelate than was the lysyl residue corresponding to resonance 5. Regardless of the exact assignment of resonances 1, 2, and 5 to lysines 24, 46, and 69 (see above), the side of the G5P dimer containing these residues was obviously closest to the chelate binding site.

Model of the Tb(DOTP)$^{5-}$·G5P Complex and Evidence for an Altered Position of Lys-24. A computer search for the Tb(DOTP)$^{5-}$ binding site on the surface of the G5P dimer was undertaken from a knowledge of the LIS values of the various ^{13}C methyl resonances. Since firm assignments were known only for resonance 4 (Lys-87) and resonance 7 (Met-1), the remaining resonances were permuted through all possible assignments consistent with previously determined constraints (i.e., Lys-24 was assigned to either resonance 1 or resonance 2, while resonance 5 was assigned to Lys-46 or Lys-69; Dick et al., 1988). The assignment of resonances 3 and 6 to either Lys-3 or Lys-7 made no difference in the calculated position of Tb(DOTP:$^{5-}$ because the LIS values for these two resonances were relatively small. The extrapolated ^{1}H LIS values were not used in the search for the Tb(DOTP)$^{5-}$ binding site.

Initial searches made it clear that no solution was possible that included the crystallographic coordinates of Lys-24, regardless of its assignment to resonance 1 or 2. That is, there were no calculated shifts as large as 2.3 ppm for any pair of symmetry-related lysyl residues for a Tb(DOTP)$^{5-}$ bound at any reasonable position on the protein. Allowing for the possibility that the position of Lys-24 was different in the solution structure of the protein than in the crystallographic structure, we omitted the coordinates of Lys-24 from further searches for the chelate binding site.

The remaining searches for the Tb(DOTP)$^{5-}$ binding site and the orientation of its symmetry axis led to solutions where both the position of the chelate and its axial orientation were nearly coincident with the 2-fold rotational axis of the G5P dimer. Two solutions for the position of the lanthanide·DOTP chelate were found, depending upon the order of assignment of Lys-46 and Lys-69 to resonances 1 and 5. Both of these assignments led to solutions that gave excellent agreement between calculated and measured LIS values for each of the ^{13}C resonances (except that of resonance 2, which was not included in the searches), as shown in Table I. No solutions were found when either Lys-46 or Lys-69 was assigned to resonance 2.

The distances between the two calculated chelate binding positions and the dimethylated amino groups are also shown in Table I. An obvious difference between the two solutions for the chelate binding site involved the distances between the chelate and the nearest lysyl residues, Lys-46 and Lys-69. In solution 1, the chelate was closer to Lys-69 than to Lys-46 (11.5 Å versus 14.1 Å), whereas in solution 2 the chelate was equidistant from these two lysines. We favor solution 1 since it gave relative distances to these two lysines that agreed with the line broadening of resonances 1 and 5 obtained upon binding of Gd(DOTP)$^{5-}$, as described above.

The binding site defined by solution 1 was within a space partially occupied by four hydrophobic residues of the G5P dimer: Leu-76, Leu-76′, Ile-78, and Ile-78′ (primed and unprimed residues are on the two symmetry-related monomers). This site is depicted in panels B and C of Figure 6, which also show the lysyl side chains at their crystallographic coordinates. We believe that Tb(DOTP)$^{5-}$ interacts with the G5P dimer by orienting its hydrophobic surface, consisting of four ethylenic groups (Figure 6A), toward a hydrophobic pocket formed by the leucyl and isoleucyl residues. This would leave the negatively charged surface, made up of four phosphonate groups, facing the bulk solvent. Such an orientation may be further stabilized by electrostatic interactions with Lys-46 and Lys-69, and possibly with Lys-24 and Arg-21 (and the residues from the symmetrically related monomer).

Discussion and Conclusions

Properties of the Methylated Gene 5 Protein. Dimethylation of a lysine ϵ-amino group does not alter the charge or greatly affect the pK_a of the lysine side chain (Means, 1977). In a previous study (Gray et al., 1984), we showed that reductively methylated fd gene 5 protein binds to fd DNA and to d[A(pA)$_6$] to give complexes that have essentially the same circular dichroism spectra as those formed with the unmodified protein. However, complexes formed with the methylated protein do have an increased salt sensitivity and are 50% dissociated at 0.15 M NaCl, whereas the normal complexes are 50% dissociated at 0.25 M NaCl. In the present work, we showed by electron microscopy that methylation of the protein did not alter its ability to form its characteristic helical complexes with DNA. This indicates that the protein–protein

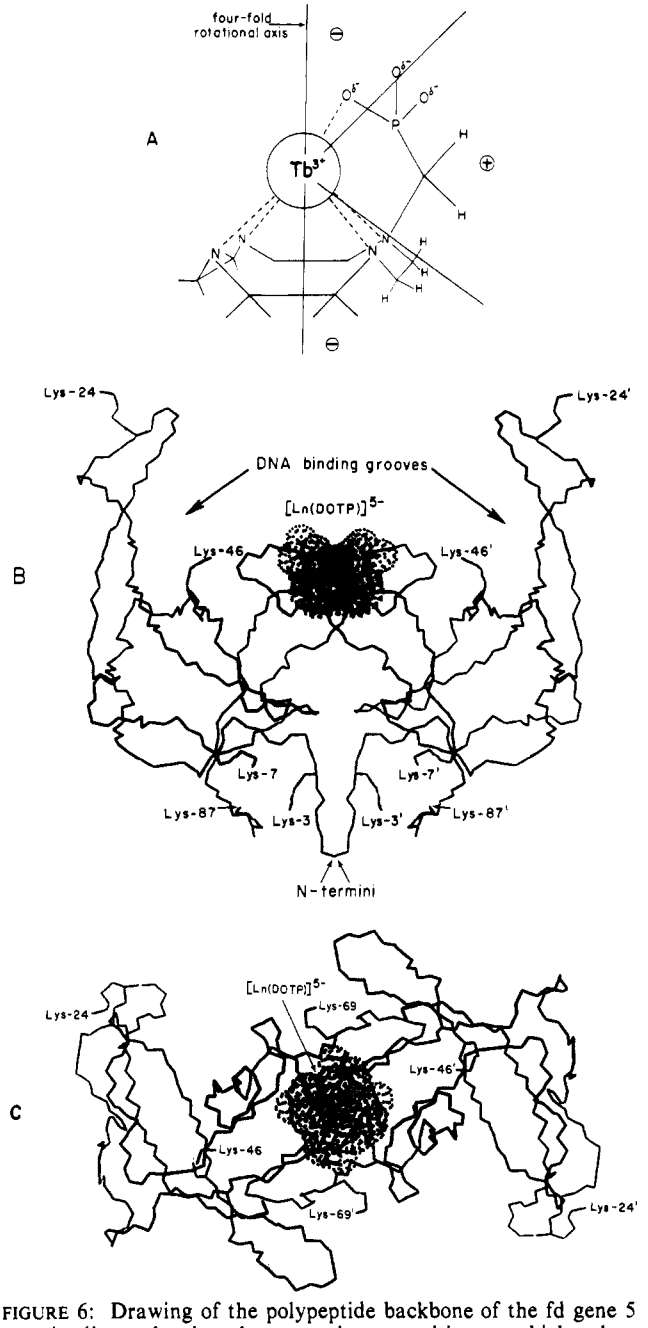

FIGURE 6: Drawing of the polypeptide backbone of the fd gene 5 protein dimer showing the approximate position at which a lanthanide·DOTP chelate probably binds. The six lysine side chains are included. Primed residues are on the symmetry-related monomer. Panel A shows a schematic of the structure of $Tb(DOTP)^{5-}$. The view is perpendicular to the 4-fold rotational axis of the chelate. Only one of the four methylenephosphonate side chains is shown for clarity. The predicted pseudocontact LIS directions are shown for each nucleus in the structure. Positive refers to shifts to higher frequency. Panel B shows the chelate (with a van der Waals surface) superimposed on the polypeptide backbone of the protein dimer at the position determined by solution 1 (Table I). The view is perpendicular to the 2-fold rotational axis of the protein. Lys-69 is obscured by the chelate in this view. Panel C shows a view down the 2-fold rotational axis of the protein from the side of the dimer that has the DNA-binding grooves. Only the three near lysyl residues are labeled on each chain. [The $Ln(DOTP)^{-5}$ in panel A is drawn on a larger scale than in panels B and C.]

cooperative interactions and the binding of the protein to DNA are at least qualitatively similar for the methylated and nonmethylated proteins.

Three Lysines Are Implicated in the Nucleic Acid Binding Function. Previous work showed that three lysyl residues of G5P, Lys-24, Lys-46, and Lys-69, are significantly protected from reductive methylation when the protein is bound to a polynucleotide, poly[r(U)] (Dick et al., 1988). In that work, resonances 1, 2, and 5 in the ^{13}C NMR spectra of the dimethylated G5P were assigned to these lysines, with Lys-24 corresponding to either resonance 1 or resonance 2. We now have shown that these same three resonances and lysyl residues were perturbed upon binding of a short DNA oligomer, $d(pA)_7$, a situation in which protein–protein interactions were minimal. Using competition experiments with a paramagnetic chelate probe, $Tb(DOTP)^{5-}$, and $d(pA)_7$, we were able to ascertain the relative positions of these resonances in the presence of the DNA oligomer alone (Figure 4). The chemical shifts of these three lysines were different from those that resulted from increasing the ionic strength (Dick et al., 1988). The present work thus supports our previous conclusion that these three lysines are directly involved in the DNA-binding function of the protein and form part of the nucleic acid binding site.

A Model for the $Tb(DOTP)^{5-}$·G5P Complex. The $Tb(DOTP)^{5-}$ chelate was useful in determining whether ^{13}C-labeled lysyl residues of the fd gene 5 protein were in the same position in solution as in the crystal structure. The fact that the $Tb(DOTP)^{5-}$ chelate has both hydrophobic and negatively charged surfaces (seen in Figure 6A) has made it particularly valuable in the study of the G5P dimer, which has a hydrophobic surface sandwiched between the two positively charged DNA-binding sites.

The orientation of $Tb(DOTP)^{5-}$ on the protein surface that was required to best account for the LIS of all the lysyl residues (except Lys-24) was one in which the hydrophobic surface of the chelate was in contact with the protein (as shown in Figure 6, panels B and C). This orientation of $Tb(DOTP)^{5-}$ induced (+) and (−) LIS values for the lysyl residues that were in agreement with the signs of the pseudocontact LIS values previously reported for the ^1H, ^{13}C, and ^{31}P nuclei of the chelate itself (Sherry et al., 1987). As shown in Figure 6A, those nuclei that make up the hydrophobic surface of $Tb(DOTP)^{5-}$ (i.e., the ethylenic carbons and three of four ethylenic protons) are shifted to lower frequencies (negative shifts), while the methylenephosphonate carbon, phosphorous, and proton nuclei shift to higher frequencies (positive shifts). Similarly, those methylated amino groups that are below the hydrophobic surface of the chelate in our model, Lys-3, Lys-7, Lys-87, and Met-1, were shifted to lower frequencies while those that are perpendicular to the 4-fold rotational axis of the chelate, Lys-46 and Lys-69, were shifted to higher frequencies (see Figure 6).

The limited ^1H data presented in Table I were entirely consistent with the proposed $Tb(DOTP)^{5-}$ binding location. The calculated LIS values from solution 1 for one of the methyl groups of Leu-83 was in good agreement with the extrapolated chemical shift. The calculated LIS values for the methyl resonances of Met-1 and Met-77 suggested that the resonance at 2.23 ppm that was shifted during the addition of $Tb(DOTP)^{5-}$ should be assigned to Met-1. Although the calculated LIS for the $C_\delta 2H$ resonance of His-64 was larger (−0.20) than our experimentally extrapolated shift (−0.03), this residue lies near the cone defined by the relation $3\cos^2\theta - 1$ (see Figure 6A), and a small rotation about the C_α–C_β or C_β–C_γ bond of His-64 could bring the calculated LIS value in line with our experimental value.

The large, positive experimental LIS value of 2.30 ppm for resonance 2 with the $Tb(DOTP)^{5-}$ chelate provided strong evidence that the corresponding lysyl residue was positioned near the methylene phosphonate groups in the chelate·G5P

complex. The dramatic line broadening observed for this resonance with the $Gd(DOTP)^{5-}$ chelate (Figure 5) also indicated that this residue was closer than any of the others. The best solutions we were able to find for our LIS data required that resonances 1 and 5 be assigned to Lys-69 and Lys-46 in some order, while resonance 2 was assigned to Lys-24 with the requirement that it be perturbed from its crystallographic position so as to be close to the chelate binding site.

Lys-24 in G5P Complexed with a Lanthanide·DOTP Chelate in Solution Is Probably Not in Its Crystallographic Position. As seen in Figures 3 and 5, the induced chemical shifts and reduced relaxation rates of the dimethylated lysines were maximal for resonance 2. This resonance was also the one most perturbed by the binding of $d(pA)_7$ (Figures 2A and 4A). Dick et al. (1988) previously suggested that this resonance might be assigned to Lys-24 on a flexible arm, partly on the basis of the facts that this resonance remained relatively sharp compared with other resonances during pH titrations and that it had one of the longest T_1 values. The assignment of Lys-24 to resonance 2 and a large change of Lys-24 from its crystallographic position were both required to allow an explanation of the LIS values observed for the methylated lysines in the present work. Consistent with this conclusion was the observation that Tyr-26 ring ^1H resonances disappeared upon addition of $Tb(DOTP)^{5-}$ to the nonmethylated protein, indicating that these resonances also were subject to large lanthanide-induced chemical shifts. Thus, both Lys-24 and Tyr-26 appeared to be close to the chelate binding site.

The crystallographic position of Lys-24 relative to the proposed chelate binding site is shown in Figure 6. Our data required that Lys-24 be closer to the chelate than either Lys-46 or Lys-69. In fact, we calculated that Lys-24 may be as close to the chelate as 6.8 Å. This would require a substantial change in the position of the β-ribbon containing Lys-24 and Tyr-26 from their crystallographic positions. Our experimental data did not pertain to the position of the β-ribbon in the uncomplexed protein, and the positions of Lys-24 and Tyr-26 in the solution structure may be similar to those in the crystal structure. We can only conclude that the β-ribbon is flexible enough so that its position is changed significantly relative to the crystal structure when the protein is complexed with $Tb(DOTP)^{5-}$ (and perhaps DNA). The possibility that the change occurs in solution is, however, consistent with our previous data (Dick et al., 1988) which showed that Lys-24 was strongly protected from methylation when the protein was bound to poly[r(U)].

Brayer and McPherson (1984) previously postulated that during DNA binding the β-ribbon (residues 15–32) moves so that Gly-23 differs by 10.1 Å from its crystallographic position; we estimate that such a movement would allow the amino group of Lys-24 to come within the required distance of our chelate. Brayer and McPherson (1984) pointed out that the position of this β-ribbon is constrained in the crystal structure of the protein, since the β-ribbon of one molecule lies in contact with the next translationally related protein molecule in the crystal. These authors also pointed out that this β-ribbon has few contacts with the remainder of the protein and that residues 21–27 exhibit high thermal factors and thus have considerable mobility even in the crystalline state (Brayer & McPherson, 1983). Although Brayer and McPherson (1984) did not specifically include Lys-24 among the residues playing a role in DNA binding, and other aspects of their proposed DNA-binding site have not proven to be correct (Kansy et al., 1986; King & Coleman 1987, 1988; de Jong et al., 1989a,b; Gray, 1989), their proposal that the β-ribbon containing Lys-24 changes from the crystal structure when the protein is complexed with DNA is substantiated by our present data.

The region of G5P that is involved in DNA binding according to NMR studies (Alma et al., 1983b; King & Coleman, 1987, 1988) is labeled in Figure 6B. In particular, Tyr-26, Leu-28, and Phe-73′ on each side of the dimer are associated with bound DNA oligomers. King and Coleman (1988) have proposed a "dynamic clamp" involving these residues and fringed by a cluster of positively charged lysines and arginines. The chemical shift that apparently occurs for the ^1H resonances of Phe-73 (3,4,5)H in the presence of $Tb(DOTP)^{5-}$, compared with the calculated LIS value (Table I), suggests that this residue may also be repositioned when the β-ribbon involved in DNA-binding arm moves toward the axis of the dimer. Thus, our data support the notion of a clamp and provide evidence that the β-ribbon with Tyr-26, Leu-28, and Lys-24 can move to bring Lys-24 well into the binding groove and possibly into intimate contact with a bound oligomer.

Acknowledgments

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Range of the Solvation Pressure between Lipid Membranes: Dependence on the Packing Density of Solvent Molecules†

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ABSTRACT: Well-ordered multilamellar arrays of liquid-crystalline phosphatidylcholine and equimolar phosphatidylcholine–cholesterol bilayers have been formed in the nonaqueous solvents formamide and 1,3-propanediol. The organization of these bilayers and the interactions between apposing bilayer surfaces have been investigated by X-ray diffraction analysis of liposomes compressed by applied osmotic pressures up to 6×10^7 dyn/cm^2 (60 atm). The structure of egg phosphatidylcholine (EPC) bilayers in these solvents is quite different than in water, with the bilayer thickness being largest in water, 3 Å narrower in formamide, and 6 Å narrower in 1,3-propanediol. The incorporation of equimolar cholesterol increases the thickness of EPC bilayers immersed in each solvent, by over 10 Å in the case of 1,3-propanediol. The osmotic pressures of various concentrations of the neutral polymer poly(vinylpyrrolidone) dissolved in formamide or 1,3-propanediol have been measured with a custom-built membrane osmometer. These measurements are used to obtain the distance dependence of the repulsive solvation pressure between apposing bilayer surfaces. For each solvent, the solvation pressure decreases exponentially with distance between bilayer surfaces. However, for both EPC and EPC–cholesterol bilayers, the decay length and magnitude of this repulsive pressure strongly depend on the solvent. The decay length for EPC bilayers in water, formamide, and 1,3-propanediol is found to be 1.7, 2.4, and 2.6 Å, respectively, whereas the decay length for equimolar EPC–cholesterol bilayers in water, formamide, and 1,3-propanediol is found to be 2.1, 2.9, and 3.1 Å, respectively. These data indicate that the decay length is inversely proportional to the cube root of the number of solvent molecules per unit volume. Thus, the decay length of the solvation pressure depends on the packing of the solvent molecules in the interbilayer space but is not strongly dependent on either the solvent's dielectric constant or the dipole moment. The magnitude of the solvation pressure, which is largest in water and smallest in 1,3-propanediol, varies with the square of the dipole potential as measured in monolayers in equilibrium with bilayers.

To bring two polar surfaces together, one must remove the intervening solvent molecules. Because the solvent molecules are ordered by the surfaces, the removal of these molecules requires energy and gives rises to a large, short-range repulsive pressure, called the solvation pressure, P_{sol}, or, if the solvent is water, the hydration pressure, P_h. For several polar and nonpolar solvents, including water, cyclohexane, and octane, Israelachvili and colleagues (Christenson et al., 1982; Israelachvili & Pashley, 1983; Israelachvili, 1985) have shown that the solvation pressure between smooth solid surfaces such as mica sheets, while overall repulsive, is an oscillatory function of the fluid spacing between the mica sheets. They found that the distance between successive minima in P_{sol} corresponds approximately to the diameter of the solvent molecule. For surfaces which are "micro-rough" or fluid, such as biological membranes and lipid bilayers, these oscillations are smeared out and the solvation pressure decreases monotonically with distance (Israelachvili & Pashley, 1983; Rand et al., 1979).

For lipid bilayers with water as the solvent, P_h has been quantitated by X-ray diffraction measurements of multilayers squeezed together by osmotic pressure. For several types of lipid bilayers immersed in water, it has been found that P_h decays exponentially with distance between bilayers (d_f) such that $P_h = P_0 \exp(-d_f/\lambda)$, where the decay length λ is on the order of 1–2 Å (LeNeveu et al., 1977; Parsegian et al., 1979; Lis et al., 1982; McIntosh & Simon, 1986; Simon et al., 1988).

Less work has been done on the solvation pressure between bilayers in liquids other than water, although a number of studies have shown that multilamellar phosphatidylcholine liposomes can be formed in nonaqueous solvents. These sol-

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