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lac Repressor Headpiece Binds Specifically to Half of the *lac* Operator: A Proton Nuclear Magnetic Resonance Study†

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ABSTRACT: The complex formation of the N-terminal domain (headpiece) of the *Escherichia coli lac* repressor and a synthetic 14-base-pair *lac* operator fragment has been investigated by ^{1}H NMR. Titration shifts in the imino-proton region of the DNA spectrum and in the aromatic region of the headpiece spectrum are examined in detail and interpreted where possible. The assignment of the resonances in the complex follows in part from the titration data and is completed by nuclear Overhauser measurements. The shift of the His-29 C-2 resonance has been used to assess the binding strength of the complex. Evidence is presented for the presence of a high-affinity site on the *lac* operator fragment ($K_D \leq 2 \times 10^{-5}$ M), which shows features in common with one of the specific binding sites on the complete *lac* operator, and for the presence of a second, nonspecific binding site with lower affinity. The influence of this second site on the interpretation of the binding data is discussed.

Since the development of the classical models for gene regulation by Jacob & Monod (1961), the *lac* operon of *Escherichia coli* has been the archetypal example of a negatively controlled operon in prokaryotic organisms. The regulation of the expression of the *lac* genes involves interaction of the *lac* repressor with *lac* operator DNA, which has been the subject of intensive research over the past two decades [for reviews, see Bourgeois & Pfahl (1976), Miller & Reznikoff (1978), and Caruthers (1980)]. Our interest in this system stems from the fact that it may serve as a model for specific DNA recognition by proteins, a process that is still ill understood at the molecular level.

The *lac* operator consists of 21-25 base pairs (see Figure 1). Many of the functional groups that are recognized by the *lac* repressor were identified by measuring changes in the stability of the specific complex upon modification of the operator sequence by a variety of chemical and genetic methods (Gilbert et al., 1975; Ogata & Gilbert, 1977; Goeddel et al., 1978; Caruthers, 1980). Additional information came from the methylation experiments of Ogata & Gilbert (1979). Binding of the *lac* repressor modifies the pattern of purine methylation in a highly characteristic manner. A very similar methylation pattern is obtained when the repressor is replaced by its N-terminal domain (residues 1-59 or 1-51, long and short headpiece, respectively). This observation implies that most of the contact sites on the protein are concentrated in its N-terminal domains, in full agreement with conclusions from studies of mutated *lac I* gene products (Adler et al., 1972; Miller, 1979).

The relatively small size of the *lac* repressor headpiece and its ready availability allow the study of its structure and interactions with operator DNA by using NMR techniques. High-resolution ^{1}H NMR studies of the headpiece (Wade-Jardetzky et al., 1979; Buck et al., 1978; Ribeiro et al., 1981a-c; Wemmer et al., 1981a,b; Arndt et al., 1981; Nick et al., 1982) and of nonspecific complex formation with poly[d(AT)] (Buck et al., 1980; Hogan et al., 1981) have already proven the great potential of NMR techniques in this area of molecular biology. We have chosen to study a chemically synthesized 14-bp[1] DNA fragment comprising half the *lac* operator (base pairs -2 to 12; see Figure 1) because there are strong reasons to believe that half of the *lac* operator is the smallest functional unit able to form a specific complex with headpiece. Several lines of evidence support the model that two subunits of the tetrameric *lac* repressor recognize the

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[1] Abbreviations: bp, base pair; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride; TCMP, trichloromethylpropane; DSS, 2,2-dimethyl-2-silapentane-1-sulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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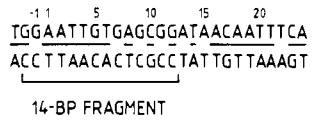

FIGURE 1: Base sequence of the *E. coli lac* operator (Gilbert & Maxam, 1973). Bars denote the symmetrical regions around base-pair GC 11. The 14-bp fragment used in this study and the numbering system employed are indicated.

two halves of the *lac* operator, thereby making use of the palindromic symmetry around base pair GC 11: (1) two subunits of the *lac* repressor in a *lac* repressor–β-galactosidase chimaera are able to form a functional complex with the *lac* operator (Kania & Brown, 1976); (2) low-angle X-ray data reveal a pairwise distribution of the four N-terminal domains over the *lac* repressor molecule (Pilz et al., 1980); (3) circular dichroism measurements confirm that the *lac* repressor can bind simultaneously two relatively short DNA fragments containing the *lac* operator (Culard & Maurizot, 1981); (4) ^{1}H NMR studies reveal that two headpiece molecules can be bound by the *lac* operator (Buck et al., 1983).

In this paper we report on a ^{1}H NMR study of the complex formation by the *lac* repressor short headpiece and the 14-bp operator fragment depicted in Figure 1. Evidence will be put forward that this complex indeed is representative of the specific complex formed with the complete *lac* operator.

Experimental Procedures

The 14-bp DNA fragment was synthesized via an improved phosphotriester approach (van Boom et al., 1976; de Rooij et al., 1979). Concentrations were calculated from the absorbance at 260 nm, using an extinction coefficient ϵ_{260} = 21.5 mL mg^{-1} cm^{-1}. This value was obtained both from a phosphorus determination (Chen et al., 1956) and a diphenylamine assay (Richards, 1974). The two methods yielded the same results within experimental error.

lac repressor was isolated from the *Escherichia coli* strain BMH 74-12, as described by Rosenberg et al. (1977). We replaced *o*-nitrophenyl fucoside (ONPF) by 5% (w/v) glucose in the "CSB" buffer.

Short headpiece (residues 1–51) (Geisler & Weber, 1977) was prepared by incubating the *lac* repressor [typically 300 mg in 10 mL 1 M Tris-HCl (pH 7.5), 30% (v/v) glycerol, 2.5 × 10^{-3} M DTE, and 1 × 10^{-3} M $CaCl_2$] with 1% (w/w) clostripain (Sigma; 71 units/mg) for 5 h at 18–20 °C. Digestion was stopped by adding 10^{-2} M EDTA and cooling in ice. Gel filtration on Sephadex G-50 [2 × 85 cm; in 0.05 M phosphate (pH 7.4), 0.4 M KCl, 10^{-3} M EDTA, 50 μg/mL PMSF, and 0.02% TCMP] was used to separate short headpiece from the other proteins. Elution was at 10–15 mL/h, and the combined fractions, containing 25–30 mg of short headpiece in about 40 mL, were concentrated to 10–15 mL in an ultrafiltration cell (Amicon, UM-2 membrane). After dialysis against 2 × 0.5 L of 0.060 M phosphate (pH 7.4), 5% glycerol (v/v), 0.5 × 10^{-3} M EDTA, 0.3 × 10^{-3} M DTE, and 50 μg/mL PMSF, the headpiece was loaded onto a phosphocellulose column (1 mL) (Arndt et al., 1981) in the same buffer. The column was washed with 10 mL of 0.06 M phosphate (pH 7.4), and headpiece was eluted with 0.05 M phosphate (pH 7.4) and 0.4 M KCl. The pooled fractions (maximally 20–25 mg/mL in headpiece) were dialyzed in a closed cell against the buffer to be used in the experiments. So that solutions could be obtained in ^2H_2O, the phosphocellulose column was washed and eluted as described above with buffers made up in ^2H_2O. This procedure yields concentrated samples in high salt that can be used for NMR measurements directly. The preparation was found to be pure and identical with that of the short headpiece described by Geisler & Weber (1977) by several techniques, including polyacrylamide gel electrophoresis [in sodium dodecyl sulfate (0.1% w/v) and urea (6 M)], isoelectric focusing (in polyacrylamide gels), and ^{1}H NMR spectroscopy. Arginine was found to be the first residue released during carboxypeptidase B treatment. Headpiece concentrations were determined spectrophotometrically at 280 nm, using ϵ_{280} = 0.88 mL mg^{-1} cm^{-1}.

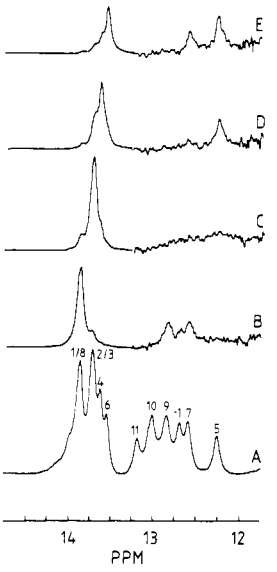

FIGURE 2: (A) Imino-proton region of the 500-MHz ^{1}H NMR spectrum of the 14-bp *lac* operator fragment recorded at 25 °C in 0.05 M phosphate (pH* 7.00), 0.5 × 10^{-3} M DSS, 0.02% (w/v) NaN_3, and 10% (v/v) ^2H_2O. A Redfield pulse sequence (see Experimental Procedures) was employed. The assignments indicated are those obtained by Zuiderweg et al. (1981), confirmed and completed by NOE difference spectroscopy (spectra B–E; see text). (B–E) NOE difference spectra obtained by preirradiation of the resolved AT resonances. The GC region (13.25–11.75 ppm) is expanded by a factor of 16 in the vertical direction. (B) Preirradiation of resonances 1 and 8; NOE's at GC −1, 7, and 9. (C) Preirradiation at resonances 2 and 3; no NOE's in the GC region. (D) Preirradiation at AT 4; NOE at GC 5. (E) Preirradiation at AT 6; NOE's at GC 5 and 7.

^{1}H NMR spectra were recorded on a Bruker HX-360 (360 MHz) or WM-500 (500 MHz) spectrometer. Chemical shifts were measured relative to DSS. The notation pH* stands for uncorrected pH-meter readings in ^2H_2O solutions. Spectra in ^1H_2O were recorded by making use of a Redfield pulse sequence (Redfield & Kunz, 1979). Further suppression of the water signal was achieved by varying the acquisition delay to bring about destructive interference for the water signal in successive cycles (Roth et al., 1980). NOE's were measured by applying a 0.3-s selective preirradiation pulse prior to data acquisition. The decoupler frequency was set alternatingly at an on- and off-resonance position, and the resulting spectra were subtracted to yield the NOE-difference spectrum. A 2–3-s relaxation delay was used between cycles. Negative NOE's are presented as positive peaks in the difference spectra.

Results

DNA Imino Protons. Figure 2A shows the low-field region of the ^{1}H NMR spectrum of the synthetic 14-bp *lac* operator fragment depicted in Figure 1. Melting studies that resulted in the assignment of the imino-proton resonances of the Watson–Crick base pairs were recently described by Zuiderweg et al. (1981). Ambiguity remained about the assignment of the resonances AT 4[2] and AT 6 due to overlap

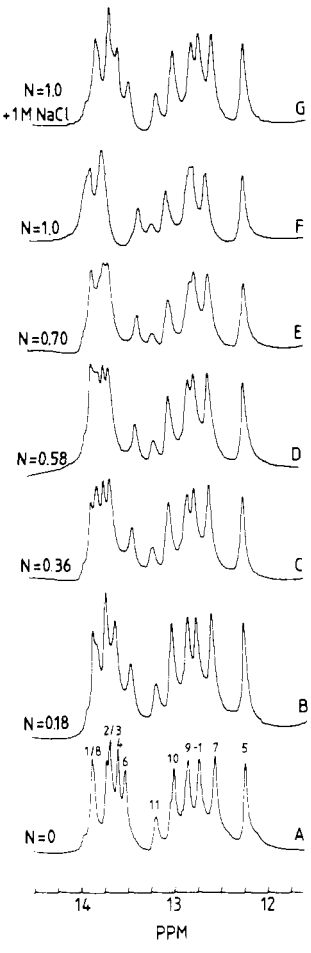

FIGURE 3: Titration of the 14-bp *lac* operator fragment with short headpiece in 10^{-2} M sodium cacodylate (pH* 6.8), 0.1 M NH_4Cl, 2×10^{-3} M DSS, 2% (v/v) glycerol, and 5% ^2H_2O, 15 °C. DNA concentration varied from 4×10^{-3} (A) to 1.6×10^{-3} M (F). (A) Imino-proton region of the 500-MHz ^1H NMR spectrum of the operator fragment. (B–F) The same region of the spectrum as a function of the headpiece/DNA molar ratio *N*. (G) Spectrum of the sample used for (F) after the addition of NaCl (1.0 M).

at temperatures where melting begins. We have now resolved this ambiguity by recording nuclear Overhauser effects (NOE's) between neighboring imino protons, which are about 3.5 Å apart in regular B-DNA (Arnott & Hukins, 1972). Figure 2B–E presents the NOE-difference spectra obtained by selective irradiation of the resolved AT resonances. From the spectra D and E the unambiguous assignments of the 13.69-ppm resonance to AT 4 and the 13.62-ppm resonance to AT 6 follow directly. The reverse experiment, where GC 7 was irradiated (spectra not shown), yields the same conclusion. The other NOE-difference spectra of Figure 2 confirm the assignments obtained by Zuiderweg et al. (1981).

Figure 3 shows how the imino-proton region of the DNA spectrum is affected by the addition of the *lac* repressor short headpiece to the DNA. Titration shifts up to a protein/DNA molar ratio of 1.8 are shown in Figure 4. The largest shifts occur in the AT region of the spectrum. AT 6 shows a 0.3 ppm upfield shift, and smaller shifts are seen for AT 4 (0.15 ppm, downfield), one of the unresolved resonances AT 2 and AT 3 (0.16 ppm, downfield), and one of the resonances AT 1 and AT 8 (0.14 ppm, upfield). In the GC region a 0.08 ppm

[2] The base-pair numbering system employed here differs from the system used by Zuiderweg et al. (1981). We follow a suggestion made by Dr. Ponzy Lu to adopt the numbering convention proposed by Ogata & Gilbert (1977).

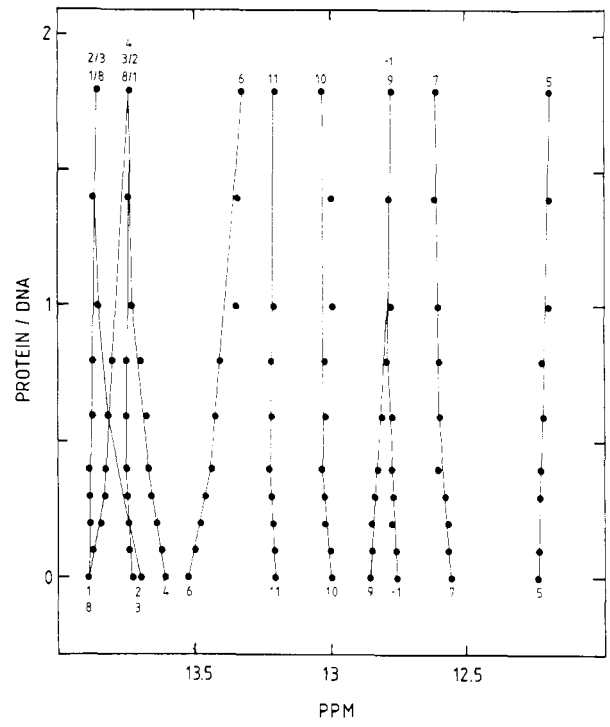

FIGURE 4: Titration shifts in the imino-proton region of the 360-MHz ^1H NMR spectrum of the 14-bp operator fragment during a titration with short headpiece. The conditions were those mentioned in Figure 3.

upfield shift occurs for GC 9, and minor (less than 0.05 ppm) shifts occur for the remaining resonances. Upon the addition of salt (1 M NaCl, spectrum G in Figure 3) the complex is dissociated to a large extent, and all resonances move toward their original positions.

Line broadening occurs for all resonances and can be explained by the higher molecular weight of the complex. The large upfield shift of resonance AT 6 is accompanied by some additional broadening due to exchange. The loss in intensity of resonance GC 11 occurs without significant shifting or additional line broadening. Zuiderweg et al. (1981) observed similar behavior for this resonance in the spectrum of the DNA fragment upon raising the temperature to about 25 °C. This suggests that headpiece binding effectively decreases the melting temperature of this next-to-terminal base pair with about 10 deg by shifting the helix–coil equilibrium (Crothers et al., 1974). This effect may well be related to the partial "unwinding" of the *lac* operator by the repressor (Wang et al., 1974). The fact that all resonances remain observable during the titration confirms the double-helical structure of the operator fragment, with intact imino-hydrogen bridges, in the complex. Large deviations from the starting (most likely B-DNA-like) conformation occurring for the fragment as a whole can be excluded, since most of the resonances show only minor shifts, while up- and downfield shifts occur to the same extent.

Headpiece Aromatic Protons. In Figure 5 the reverse titration is presented. We monitored the aromatic region of the headpiece spectrum and increased the concentration of the *lac* operator fragment. Figure 6 shows the titration shifts up to a DNA/protein molar ratio of 2. The assignment of these resonances to the four tyrosines (7, 12, 17, and 47) and the one histidine residue (29), as indicated in Figure 5, had been obtained previously by Ribeiro et al. (1981a) and Arndt et al. (1981). Two of the eight tyrosine doublets show a relatively large downfield shift. One belongs to the Tyr-17 3,5 protons

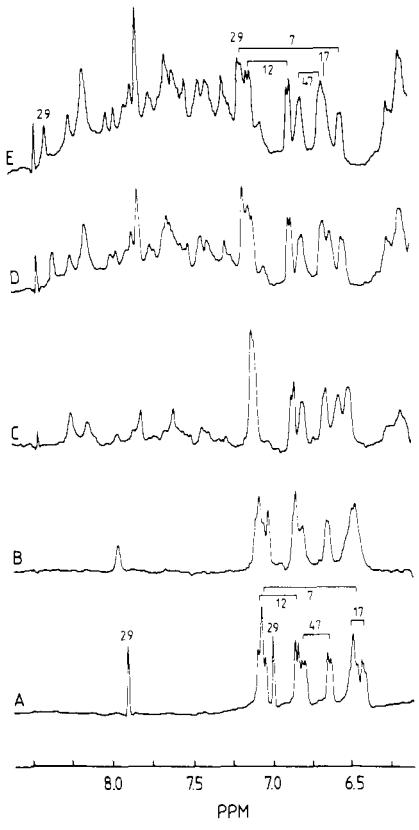

FIGURE 5: Titration of short headpiece (1.7×10^{-3} M) with the 14-bp *lac* operator fragment at 23 °C in 0.06 M phosphate (pH* 7.30), 0.5 $\times 10^{-3}$ M EDTA, 0.3×10^{-3} M DTE, and 0.5×10^{-3} M DSS in ^2H_2O. (A) Aromatic region of the 360-MHz ^{1}H NMR spectrum of short headpiece. The assignment of these resonances to the 2,6 and 3,5 protons of the four tyrosines and the His-29 C-2 and C-4 protons is indicated (Ribeiro et al., 1981a; Arndt et al., 1981). (B–E) The same region at DNA/protein ratios of 0.1, 0.7, 1.5, and 2.0, respectively.

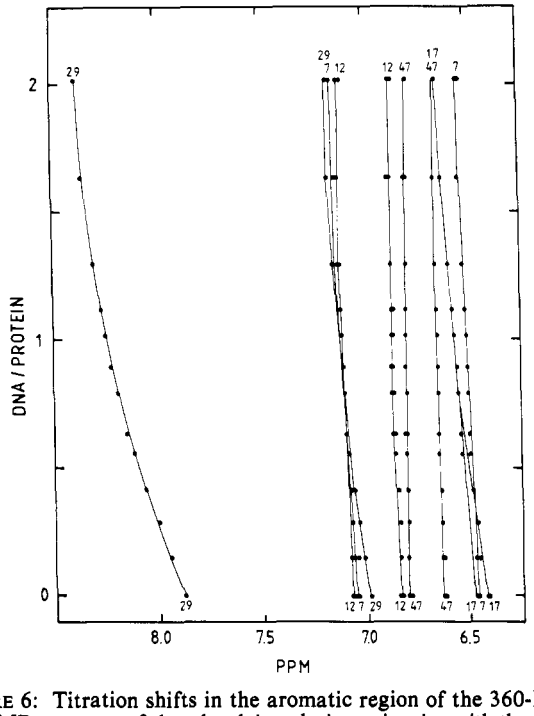

FIGURE 6: Titration shifts in the aromatic region of the 360-MHz ^{1}H NMR spectrum of short headpiece during a titration with the 14-bp operator fragment. The conditions were those mentioned in Figure 3.

at 6.41 ppm. The other to either the Tyr-17 2,6 protons or the Tyr-7 3,5 protons around 6.47 ppm, which cannot be

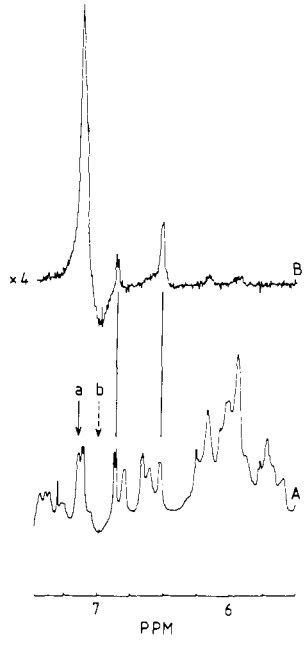

FIGURE 7: 500-MHz ^{1}H NMR spectra of the complex of short headpiece and the 14-bp *lac* operator fragment (DNA/protein molar ratio 1.6) in 0.02 M phosphate (pH* 7.35), 0.1 M NaCl, and 10^{-3} M DSS in ^2H_2O, 25 °C. (A) Aromatic region of the spectrum (resolution enhanced by a Lorentzian-to-Gaussian transformation). (B) NOE difference spectrum obtained by preirradiation of the Tyr-7 and (less effectively) Tyr-12 2,6-proton resonances [indicated by the arrow marked (a); the off-resonance position was at (b): see Experimental Procedures]. NOE's at 6.87 and 6.53 ppm, corresponding to Tyr-3,5 proton positions, are indicated.

decided from the titration data alone. We resolved this point by making use of the strong intraresidue NOE's that occur between the 2,6 and 3,5 protons of tyrosine residues. When the low-field doublets belonging to the 2,6 protons of Tyr-7 and -12 around 7.1 ppm are irradiated, strong NOE's are expected at the positions of the corresponding 3,5 protons. We performed this experiment in the presence of a slight excess of the 14-bp operator fragment, and the result is shown in Figure 7. In this way the 6.53-ppm doublet is assigned to the Tyr-7 3,5 protons in the complex. It follows that both the 2,6 and 3,5 protons of Tyr-17 show large (0.18, and 0.25 ppm, respectively) downfield shifts, finally overlapping the Tyr-47 3,5 doublet at 6.66 ppm. Smaller downfield shifts occur for the Tyr-7 doublets at 6.46 and 7.05 ppm, while the doublets of Tyr-12 and Tyr-47 hardly show any shift.

Incidently, Figure 7 also shows weak NOE's at 6.17 and 5.94 ppm, where DNA lines resonate. These and other protein–DNA NOE's will be the subject of a forthcoming paper.

The most salient feature of the titration is the large (0.5 ppm) downfield shift of the His-29 C-2 resonance, concomitant with extensive exchange broadening. This shift and that of the His-29 C-4 proton suggest that protonation of His-29 occurs during the titration. Since the pH was carefully kept constant, a rise in the pK_a of this residue must have occurred. To check this possibility, we determined the pK_a of His-29 in the presence of a small excess of the *lac* operator fragment. Figure 8 shows the pH dependence of the chemical shifts of the His-29 C-2 and C-4 and those of the Tyr-17 protons. A pK_a of 7.19 (vs. 6.80 in the free headpiece) can be calculated from the His-29 curves, with a Hill coefficient of 1.0. However, the change in chemical shift of the Tyr-17 resonances suggests that some dissociation of the complex occurs at the higher pH values. We shall discuss this point in further detail below.

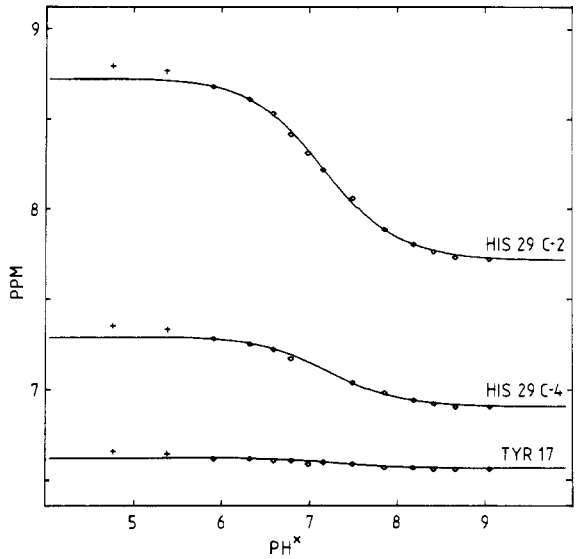

FIGURE 8: pH dependence of the His-29 C-2 and C-4 and the Tyr-17 2,6- and 3,5-proton chemical shifts in the complex of short headpiece with the 14-bp *lac* operator fragment (DNA/protein molar ratio 1.3) in 0.02 M phosphate, 0.1 M NaCl, 0.5×10^{-3} M DSS, and 0.02% (w/v) NaN_3 in ^2H_2O, 23 °C. The solid lines are the best fits (by a least-squares analysis) to the experimental points indicated by (◇). pK_as of 7.19 and 7.22 (with a Hill coefficient of 1.0) were used for the His-29 C-2 and C-4 curves, respectively.

Headpiece Aliphatic Protons. In the aliphatic region of the headpiece spectrum chemical shift changes are seen for the methyl resonances of Met-42 (0.07 ppm, downfield) and the three high-field doublets that were assigned to Leu-6 and -45 methyl protons (Ribeiro et al., 1981b). The 0.33-ppm doublet shows a 0.1-ppm downfield shift, while the 0.45- and 0.48-ppm doublets collapse and shift to 0.52 ppm.

Discussion

DNA Imino Protons. The imino-proton region of nucleic acid NMR spectra is now easily accessible by the development of efficient methods to suppress the enormous water signal (Redfield & Kunz, 1979). Redfield et al. (1981) and Patel et al. (1982) showed that NOE's can be measured between neighboring imino protons in double-stranded regions of nucleic acids. Wagner & Wüthrich (1979) presented a three-spin approximation to calculate truncated driven NOE's in isotropically tumbling molecules. By combining their theory with that of Woessner (1962) to take into account the anisotropic motion of the DNA, we estimated NOE's around −3% for neighboring imino protons (about 3.5 Å apart) in our 8500-dalton DNA fragment at a frequency of 500 MHz. This corresponds well to the magnitude of the NOE's shown in Figure 2. Measurement of these small NOE's provides a powerful tool for obtaining assignments in this region of the DNA spectrum, as demonstrated in this example.

Having these assignments we shall discuss the titration shifts shown in Figure 3. The importance of base-pair GC 5 in the *lac* repressor–*lac* operator interaction is clear both from genetic studies (Gilbert et al., 1975) (replacement of GC 5 by an AT base pair was reported to be an operator-constitutive mutation) and from methylation experiments (Ogata & Gilbert, 1979) (methylation of the guanine N-7 in base-pair GC 5 is strongly suppressed in the presence of the *lac* repressor or its headpiece). However, hardly any shift is observed for the GC 5 imino proton in the titration shown in Figure 3. Moreover, base-pair AT 6, while showing the largest shift in the titration with short headpiece, is completely silent in genetic and chemical modification studies (Gilbert et al., 1975; Ogata & Gilbert, 1977; Caruthers, 1980). We propose a solution of these apparent contradictions based on the notion that the chemical shifts of the AT 6 and AT 4 imino protons are strongly affected by shielding effects originating from GC 5. Hence, a change in orientation of GC 5 under the influence of protein side chains may explain the shifts observed for the AT 4 and AT 6 imino protons. Thus, we calculated the effect of a rotation of the guanine ring about an axis through its C-4 and N-9 atoms on the chemical shift of the AT 4 and AT 6 imino protons (see Figure 9). This rotation leaves the position of the imino proton of GC 5 almost unaffected, explaining the absence of titration shifts for this proton. Up- and downfield shifts are predicted for the AT 4 and AT 6 protons, respectively, upon clockwise rotation of the guanine (see Figure 9) in accordance with the experiment. Hence, a conformational change of this type would account for both the NMR results and the other available data described above. This example illustrates that the chemical shifts of imino protons, which are located in the center of the DNA double helix, are very sensitive to changes in the DNA structure. They are probably not much affected by shielding or deshielding effects induced by (nonintercalating) ligand molecules.

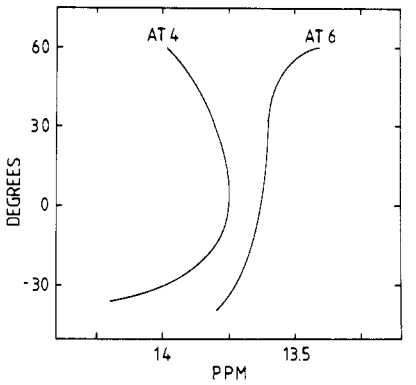

FIGURE 9: Chemical shift variation of the AT 4 and AT 6 imino protons under the influence of a rotation of the guanine ring of base-pair 5. Zero rotation corresponds to the B-DNA structure (Arnott & Hukins, 1972), and rotation is around an axis through the N-9 and C-4 atoms of the guanine. Positive dihedral angles correspond to a clockwise rotation, when viewed from the C-4 in the direction of the N-9 atom. Chemical shifts were calculated as described by Zuiderweg et al. (1981).

Headpiece Protons. Tyr-47 is only slightly accessible for N^3-(carboxymethyl)lumiflavin in a photo-CIDNP experiment (Buck et al., 1980) and is essential for maintaining the native protein structure (Arndt et al., 1981; Ribeiro et al., 1981a). Therefore the resonance positions of its protons will mainly reflect the structure of its protein environment. The titration of Figure 5 gives no indication of a conformational change in the environment of Tyr-47, although the 0.07-ppm downfield shift of Met-42 suggests that this residue has changed its position with respect to Tyr-47. Tyr-12 is accessible to the flavin dye in photo-CIDNP experiments, both in the absence and presence of poly[d(AT)] (Buck et al., 1980). Its behavior in the titration with the *lac* operator fragment (Figure 5) supports the conclusion that this residue is not involved in DNA binding.

More interesting are the residues Tyr-7 and -17, which are accessible to the flavin dye, but not so in the presence of poly[d(AT)] (Buck et al., 1980). They undergo large shifts in the titration shown in Figure 5. Their possible involvement in the interaction of the *lac* repressor headpiece with DNA makes an interpretation of their titration behavior in structural terms desirable. There is some evidence that these two residues are stacked (Nick et al., 1982), causing their protons (except

the Tyr-7 2,6 protons) to resonate at higher field than those of the free amino acid. In the presence of the operator fragment, however, these protons will sense not only the mutual orientation of the tyrosine rings but also the DNA environment. So, although it is likely that the observed shifts of Tyr-7 and -17 resonances are at least partially caused by a change in the relative orientation of these residues, it is impossible to extract the details of this structural change from chemical shift data alone.

The large shifts of the His-29 C-2 and C-4 resonances result from protonation of this residue during the titration with the operator fragment. To avoid overlap problems, we measured the pK_a of this residue in the presence of only a small excess of DNA (see Figure 8). Saturation is not complete under these conditions. Moreover, the dissociation constant of the complex is pH dependent due to the pK_a shift of His-29 (see below), so that dissociation of the complex occurs to a larger extent at the higher pH values, as monitored by the chemical shift of the Tyr-17 resonances. Therefore the pK_a of His-29 in the complex is higher than the measured value of 7.19. From the available data a reasonable estimate can be made, which yields a pK_a of 7.30 ± 0.05, implying a rise of 0.5 unit upon complex formation.

The pH dependence of the dissociation constant around pH 7 is described by

$$K_D = K_D^0 \frac{1 + 10^{pH-pK_a^H}}{1 + 10^{pH-pK_a^{OH}}}$$

in which K_D^0 is the dissociation constant of the complex at low pH values (relative to the lowest pK_a value). pK_a^H and pK_a^{OH} stand for the pK_a values of the His-29 residue in the isolated headpiece and in the complex, respectively. It follows that the dissociation constant increases by a factor of 3 on going from low to high pH values. This corresponds to 2.8 kJ/mol, which can be ascribed to the free energy change that accompanies the formation of an ionic contact by His-29 in the complex, most likely with the DNA phosphate backbone. Photo-CIDNP data revealed the accessibility of His-29 to the flavin dye in the absence of poly[d(AT)] only (Buck et al., 1980), in good agreement with the conclusions reached here.

Finally, we think it is important to mention that most of the resonances remain unaffected, which means that the protein structure does not undergo extensive changes upon complex formation. This conclusion is important since it implies that structural studies on the isolated headpiece may well be extrapolated to the headpiece–operator complex, as was demonstrated recently for the *cro* repressor from bacteriophage λ (Anderson et al., 1981).

Evidence for Specific Binding. Our choice to study a 14-bp DNA fragment comprising half of the *lac* operator sequence enabled us to analyze the above ^{1}H NMR titration data in detail. The use of this relatively small DNA fragment, however, raises the question whether the complex we study is indeed representative of the specific *lac* operator–*lac* repressor complex. Comparison of our titration data with those of Nick et al. (1982) is one way to answer this important question. Nick et al. studied the complexation of a slightly larger headpiece molecule (residues 1–56) with a 36-bp DNA fragment containing the complete *lac* operator sequence. Although the high molecular weight of this DNA fragment impeded the complete analysis of their ^{1}H NMR data, they were able to show that the downfield shift of the Tyr-17 3,5 protons only occurs in a titration with operator DNA and is not observed in a titration with random DNA of similar size. Our 14-bp DNA fragment induces the same downfield shift of the Tyr-17 3,5 protons, and we regard this as evidence that we are indeed studying the specific complex of *lac* repressor headpiece with operator DNA.

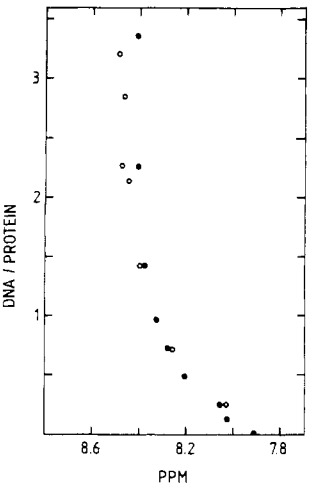

FIGURE 10: Chemical shift variation of the His-29 C-2 proton during a titration of short headpiece with the 14-bp *lac* operator fragment in 0.05 M phosphate, 0.5 × 10^{-3} M DSS, and 0.02% (w/v) NaN_3, pH* 7.05, in ^2H_2O, 25 °C. Headpiece concentration varied from 1.3 × 10^{-3} to 0.88 × 10^{-3} M (closed circles) and from 0.21 × 10^{-3} to 0.16 × 10^{-3} M (open circles).

Other evidence comes from a comparison of our titration of the operator fragment with short headpiece (see Figure 3) with a similar titration performed by Buck et al. (1983). They titrated a DNA restriction fragment, containing two copies of the complete *lac* operator in tandem, with short headpiece and observed the same changes (among others) in the imino-proton region as those observed in our titration. Most notably, the shift pattern of the AT 4 and AT 6 resonances (see Figure 3) is also clear in this larger model system.

So it seems safe to conclude that the *lac* repressor short headpiece binds to our 14-bp *lac* operator fragment in the same way as it binds to the corresponding half of the complete *lac* operator.

Evidence for Nonspecific Binding. The titrations presented in this paper were carried out at an ionic strength not exceeding 0.1 M. Under these conditions headpiece sticks to phosphocellulose, and hence, the possibility of nonspecific complex formation through the DNA phosphate backbone must be taken into account. The titrations shown in Figure 3 and Figure 5 gave us no reason to consider more than one binding site for headpiece on the DNA fragment. So, if additional, nonspecific binding does occur, the contribution of such complexes to the observed signal (e.g., the chemical shift of the His-29 C-2 resonance) cannot be large, since this would be apparent from the observed stoichiometries. The presence of such silent sites can only be demonstrated by performing the titrations at different concentrations of the species involved. Figure 10 shows the result of two titrations of short headpiece with the DNA fragment. From the titration performed with 1 mM headpiece (closed circles), an apparent dissociation constant of 1.2 × 10^{-4} M can be derived. The other curve in Figure 10 (open circles) was obtained with 0.17 mM headpiece. Calculations showed (Figure 11A) that such a 6-fold dilution would affect the binding curve significantly, if a simple 1:1 binding equilibrium is involved. This, however, is not observed experimentally (Figure 10): very similar binding curves were obtained at both concentrations.

Obviously, we must be observing stoichiometric binding, since this would make the binding curves insensitive to dilution. However, this is hard to reconcile with the shape of the binding

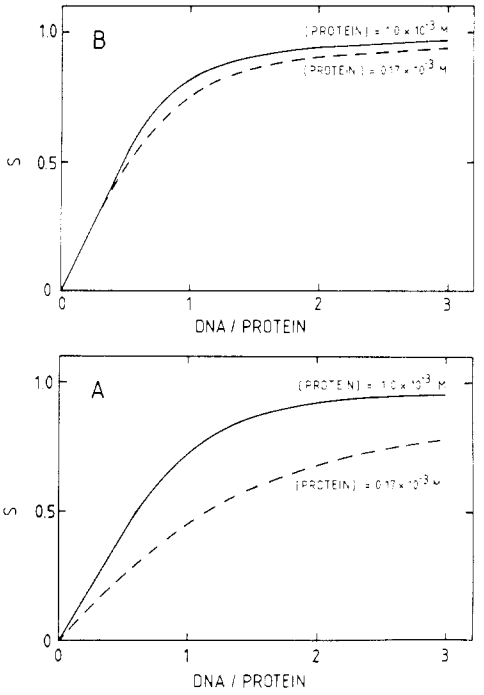

FIGURE 11: Calculated binding curves on the basis of a one-site (A) or a two-site (B) model at two different concentrations of the protein. For parameters and formalism, see the text and Appendix.

curves in Figure 10 and the dissociation constants apparent from them (1.2×10^{-4} M and 2.0×10^{-5} M; closed and open circles, respectively), which seem to show that we are not in the regime of stoichiometric binding at the concentrations employed. This dilemma can be resolved if we drop the assumption of 1:1 binding and include additional, weaker binding sites on the DNA. Figure 11B shows the result of some model calculations (for details, see the Appendix).

The curves in Figure 11B were calculated on the basis of a two-site model with intrinsic dissociation constants of 1.0 $\times 10^{-5}$ M and 1.0×10^{-4} M for the high- and low-affinity sites, respectively. As discussed above, only saturation of the high-affinity site is assumed to contribute to the signal. This very simple model already predicts the effects observed, namely, a relatively high apparent dissociation constant, but insensitivity of the titration curve to dilution. We also performed calculations on the basis of more realistic models, e.g., one in which two overlapping sites are assumed on both sides of the DNA, one of these four sites being of high affinity. Such models, which account for the interdependence of the specific and nonspecific binding processes in a more realistic manner, can explain the experimental results of Figure 10 equally well. However, it is impossible to extract the parameters of the nonspecific binding process from the data collected thus far. In view of the important role of nonspecific complex formation in the in vivo system (Winter et al., 1981), the effects observed here certainly deserve further attention.

It is clear from all models tested that the apparent dissociation constant overestimates the intrinsic dissociation constant of the high-affinity site considerably, the discrepancy being larger at higher concentrations. This is caused by the fact that a significant fraction of the headpiece molecules that are assumed to be free for the evaluation of the dissociation constant is in fact involved in low-affinity complexes. This fraction increases with the concentration of the DNA, and hence the apparent dissociation constant increases with this concentration as well. We therefore present the dissociation constant of 2×10^{-5} M, obtained at the lower concentration in the experiment of Figure 10, as an upper limit for the dissociation constant of the specific complex. This value is in agreement with that obtained by Ogata & Gilbert (1979) from the protecting effect of short headpiece on the methylation of the guanine in base-pair GC 5. Since both these values pertain to the same part of the *lac* operator, this provides additional evidence that the complex of one headpiece molecule with half of the *lac* operator is indeed a functional unit of the specific complex of the *lac* repressor and the *lac* operator.

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Appendix

The binding curves presented in Figure 11B were calculated on the basis of a model in which two independent sites exist on the DNA. The following equations apply:

$$c_1 = [\mathrm{DNA}]_f\left(\frac{c_f}{K_1} + \frac{c_f^2}{K_1K_2}\right)$$

$$c_2 = [\mathrm{DNA}]_f\left(\frac{c_f}{K_2} + \frac{c_f^2}{K_1K_2}\right)$$

$$c_{tot} = c_1 + c_2 + c_f \qquad (1)$$

in which c_1 and c_2 stand for the concentrations of ligand (headpiece) bound to sites 1 and 2 (with intrinsic dissociation constants K_1 and K_2), respectively. The subscript f denotes the unbound species and tot the sum of bound and unbound species. At a certain value of c_{tot}, $[\mathrm{DNA}]_f$ is varied, and the corresponding value of c_f is found by solving eq 1. The individual titration points are then calculated by using

$$[\mathrm{DNA}]_{tot} = [\mathrm{DNA}]_f\left(1 + \frac{c_f}{K_1} + \frac{c_f}{K_2} + \frac{c_f^2}{K_1K_2}\right)$$

and the signal observed is calculated by

$$S = \epsilon_1 c_1 + \epsilon_2 c_2$$

which is normalized with respect to its maximal value at high DNA concentrations:

$$\bar{S} = S/S_{max}$$

ϵ_1 and ϵ_2 describe the contributions of the two types of complexes to the signal. Figure 11A was constructed by using $\epsilon_2 = 0$, meaning that only site 1 is assumed to contribute to the observed signal.

Registry No. 14-base-pair *lac* operator fragment, 83846-39-3.

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