Nonspecific Interaction of the *lac* Repressor Headpiece with Deoxyribonucleic Acid: Fluorescence and Circular Dichroism Studies†

Manfred Schnarr,‡ Maurice Durand, and Jean-Claude Maurizot*

ABSTRACT: The nonspecific interaction of the short headpiece, the NH_2-terminal domain of the *lac* repressor, with natural DNA and alternating polydeoxynucleotides has been studied by means of fluorescence and circular dichroism. The important quenching of the intrinsic tyrosine fluorescence of the headpiece upon complexation has been used for the determination of the binding isotherms under various environmental conditions. By comparison with theoretical binding curves, we have determined a physical site size of three base pairs. The "perturbated" site size as determined from circular dichroism measurements (about four base pairs) is slightly greater. As in the case of the entire *lac* repressor, the interaction is strongly ionic strength dependent. The plots log K_{obsd} as a function of log [NaCl] are linear for salt concentrations greater than 50 mM for the interaction with DNA and greater than 25 mM for the interaction with poly[d(G-C)]. From the slopes of the linear parts of these plots, we determine a number of three electrostatic interactions, assuming no anion release from the protein upon complexation. This value is independent of pH. On the contrary, the association constant K_{obsd} depends on pH. Complexation requires the protonation of one titrating group of the headpiece with a pK value of 6.7 ± 0.3, probably the residue histidine-29. The finding is in favor of the idea that the *lac* repressor interacts with DNA via two headpieces as earlier work has shown that the interaction of the *lac* repressor with DNA requires the protonation of two groups of the protein.

The *lac* repressor is a tetrameric protein with 360 amino acids per subunit, which negatively controls the expression of the structural genes of the *lac* operon by binding to the *lac* operator (Müller-Hill, 1975; Bourgeois & Pfahl, 1976). Upon limited proteolysis of *lac* repressor in high ionic strength buffer, one obtains four small N-terminal "headpieces" (residues 1–51, 1–56, or 1–59, depending on the protease used and on the duration of the hydrolysis) and a tetrameric "core" (Geisler & Weber, 1977, 1978). The core exhibits full inducer binding activity whereas the headpieces constitute the major, if not the entire, part of the DNA binding domain of the *lac* repressor. Within the intact *lac* repressor, the headpieces are very mobile as shown by NMR[1] measurements (Buck et al., 1978; Wade-Jardetzky et al., 1979) but nevertheless stabilize the core entity against denaturation by urea (Schnarr & Maurizot, 1981), pointing to the "hinge region" (Müller-Hill, 1975) between residues 51 and 59 as the probable contact zone between the headpieces and the core. These contacts seem to be loosened upon inducer binding (Schnarr & Maurizot, 1981).

The core and headpiece domains, once isolated, maintain essentially the same structures as within the entire *lac* repressor. This has been shown by a multitude of experimental methods like NMR (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981a,b), differential absorption of the aromatic residues (Matthews, 1974), tritium–hydrogen exchange kinetics (Ramstein et al., 1979), circular dichroism (Schnarr & Maurizot, 1981), or infrared spectroscopy (Schnarr & Maurizot, 1980).

Much attention has been focused on the elucidation of the structure of the isolated headpiece by spectroscopic methods. It has been shown that the single histidine and all the tyrosine residues, except that at position 47, are situated at the surface of the protein and that tyrosine residues 7 and 17 are very close to each other (Arndt et al., 1981). About half of the 51 residues of the short headpiece are involved in α-helical structure, whereas no extended β-sheet of more than two strands may be present (Schnarr & Maurizot, 1982a). By small-angle neutron scattering, it has been shown that the headpiece has an elongated shape, with a long axis of about 50 Å and a cross-sectional diameter of 15–20 Å (Charlier et al., 1981).

The isolated headpiece interacts nonspecifically with nonoperator DNA (Geisler & Weber, 1977; Jovin et al., 1977), preferentially with double-stranded DNA (Durand et al., 1983), specifically with operator-containing DNA fragments (Nick et al., 1982; Pörschke et al., 1982), inducing a very similar pattern of modification of the *lac* operator by dimethyl sulfate as the entire *lac* repressor (Ogata & Gilbert, 1978, 1979). One operator fragment interacts specifically with two headpiece molecules as we have shown by circular dichroism and fluorescence measurements (Culard et al., 1982).

In this paper, we present results on the nonspecific interaction of the *lac* repressor headpiece with nucleic acids, including the determination of the site size and the number of electrostatic interactions, as well as the dependence on temperature and pH. Our results are in favor of the idea that the *lac* repressor interacts with DNA even in the nonspecific binding mode via two headpieces.

Materials and Methods

The *lac* repressor from *Escherichia coli* BMH 493 was purified as described by Rosenberg et al. (1977). The N-terminal tryptic headpiece was prepared by incubation of the *lac* repressor (ca. 7 mg/mL) in a buffer containing 1 M Tris-HCl, pH 7.5, 30% glycerol, and 0.01 M mercaptoethanol with trypsin [beef, treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] as described by Geisler & Weber (1977). Proteolysis was stopped after 3 h by the addition of soybean

† From the Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orleans Cedex, France. *Received November 29, 1982.* This work was supported by a grant from the D.G.R.S.T. (81E1213) and Le Comité du Loiret de la Ligue Nationale Française contre le Cancer.

‡ Present address: Institut de Biologie Moléculaire et Cellulaire, C.N.R.S., Laboratoire de Biophysique, 67084 Strasbourg Cedex, France.

[1] Abbreviations: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-3563$01.50/0

trypsin inhibitor. Tryptic digestion for 3 h produces a short headpiece (1–51) as shown by Ogata & Gilbert (1978). The headpiece was isolated by gel filtration on a Sephacryl S-200 column, followed by a phosphocellulose column step. The headpiece, identified by its characteristic tyrosine fluorescence, eluted as a single peak, indicating the production of virtually pure short headpiece. The absence of any tryptophan-containing impurity was shown by fluorescence spectroscopy. Purity of the headpiece was further assessed by NaDodSO$_4$–polyacrylamide gel electrophoresis. In a last step, the ionic strength of the headpiece stock solution (final concentration 7×10^{-4} M) was adjusted by dialysis against a 40 mM potassium phosphate buffer. This purification procedure differs slightly from that we used previously (Schnarr & Maurizot, 1982a,b). It leads to the same ellipticity value at 220 nm of about 1.65×10^{-4} deg·cm^2·dmol^{-1} (at intermediate ionic strength) as determined from circular dichroism (CD) measurements.

Fluorescence measurements were done with a Jobin-Yvon spectrofluorometer in 5 × 5 mm cells. In general, the circular dichroism measurements were done with the same cell and sample, using a Jobin-Yvon Mark III dichrograph. Each CD spectrum was run at least twice, and we currently checked for eventual base-line shifts. For both types of measurements, the temperature was adjusted with a circulating water bath and held constant within ±1°C.

We used a homogeneous DNA of about 1100 base pairs, the nonoperator part of the plasmid pBR 345 after excision of the operator-containing fragment, to study the interaction with the headpiece [for details of the purification, see Culard & Maurizot (1981)]. This DNA fragment has a G-C content of about 54%. The poly[d(G-C)] was purchased from P-L Biochemicals and the poly[d(A-T)] from Boehringer (Mannheim). Both were used without further purification. All nucleic acid samples were extensively dialyzed against our low ionic strength binding buffer: 10^{-3} M potassium phosphate–10^{-4} M EDTA, pH 7.25. Concentrations were determined from absorption measurements by using molecular extinction coefficients of $\epsilon_{280} = 4800$ L·mol^{-1}·cm^{-1} for the headpiece, $\epsilon_{255} = 7100$ L·mol^{-1}·cm^{-1} for poly[d(G-C)], $\epsilon_{260} = 6650$ L·mol^{-1}·cm^{-1} for poly[d(A-T)], and $\epsilon_{260} = 6630$ L·mol^{-1}·cm^{-1} for DNA, as determined by interpolation from the values determined by Felsenfeld & Hirschmann (1965).

The pH-dependent measurements were done by adding small aliquots of NaOH or HCl (0.1 or 0.05 N) to a headpiece–DNA sample in 10^{-3} M potassium phosphate–10^{-4} M EDTA. To avoid an eventual contamination with fluorescent impurities from the pH electrode, the pH measurements were done in parallel on a second sample of exactly the sample composition as that used for the fluorescence and circular dichroism measurements.

Theory

Our interpretation of the ionic strength and pH dependence of the observed equilibrium association constants follows the theory developed by Record et al. (1976), further extended and applied to the nonspecific interaction of the *lac* repressor with DNA by the same authors (de Haseth, 1977). Using binding theory (Wyman, 1964; Schellman, 1975) and the polyelectrolyte theory of Manning (1969), they deduced the following equations:

$$-\left(\frac{\partial \log K_{obsd}}{\partial \log [M^+]}\right) = Z\psi + K \qquad (1)$$

at constant pH and temperature, and

$$-\left(\frac{\partial \log K_{obsd}}{\partial pH}\right) = \frac{r}{1 + K_H[H^+]} \qquad (2)$$

K_{obsd} is the observed equilibrium association constant, $[M^+]$ the monovalent cation concentration, Z the number of ion pairs between protein and nucleic acid, ψ (known to be 0.88 for double-helical DNA) the fraction of monovalent cations bound per DNA phosphate, K the number of anions released by the protein on binding to DNA, r the number of groups of the protein that must be protonated above their normal pK to allow for binding to DNA, K_H the intrinsic association constant of these groups, and $[H^+]$ the H^+ ion concentration.

Equation 1 may be used (in the absence of independent information on anion release) to determine the upper limit of the number of electrostatic interactions involved in the association reaction from the slope of the straight line obtained by plotting log K_{obsd} against log $[M^+]$. The presence of anion release should introduce curvature into such a plot. Equation 2 allows the determination of the number of groups of the protein that are protonated upon complex formation, assuming that the DNA does not undergo titration in the pH range of interest (pH 6–9). At sufficiently alkaline pH, log K_{obsd} is predicted to be a linear function of pH with a slope of $-r$, since $K_H[H^+] \ll 1$. At the point where $K_H[H^+] = 1$, which is the pK of the titrating groups, the magnitude of the slope is half its alkaline value. At sufficiently low pH, K_{obsd} becomes independent of pH, because all participating groups are fully protonated. In this model, the protonation is considered as a prerequisite for complex formation. It implies that the number of ionic interactions is pH independent.

Another type of pH dependence is described by the "titration curve model" (Lohman et al., 1980a,b). In this model, the unprotonated species binds to the DNA too, however, with a smaller affinity. At alkaline pH value, the binding becomes pH independent, whereas the number of ionic interactions depends on pH. The titration curve model applies to the interaction of pentalysine with DNA (Lohman et al., 1980a,b) and probably to the specific interaction of the *lac* repressor with operator DNA (Barkley et al., 1981).

Theoretical binding isotherms to fit the exponential binding curves were calculated according to the theory developed by McGhee & von Hippel (1974) to describe the binding of large ligands to a homogeneous one-dimensional lattice (the nucleic acid). For a given parameter set of site size n (in base pairs), association constant K, and total DNA concentration P_0 (in base pairs), the binding density ν (in moles of bound ligand per mole of base pairs) was varied stepwise ($0 \leq \nu \leq 1/n$), and each time, the corresponding total ligand concentration was calculated from

$$L_f = \frac{\nu[1 - (n-1)\nu]^{n-1}}{K(1 - n\nu)^n} \qquad (3)$$

and

$$L_b = \nu P_0 \qquad (4)$$

with L_f and L_b being the free and bound ligand concentrations, respectively. Equation 3 describes a noncooperative type of binding. About 200 different binding isotherms for very different parameters sets were calculated with a CII 10070 computer and plotted directly on the computer listing to facilitate for comparison with the experimental binding isotherms.

It is important to define the physical site size since it will be seen that it may differ from the length of the DNA that is perturbated by the binding of one headpiece. Assuming that

the headpiece binds the DNA with a single geometry relatively to the grooves and to the phosphodiester backbones, the positions of two headpieces on the DNA are related by a helical transformation. This transformation is the product of a translation of kl and a rotation of $k\omega$, where l and ω are respectively the distance and the angle between two lattice motives and k is an integer. In the B form l = 3.4 Å and ω = 36°. The smallest number of k that is compatible with the sterical restrictions and eventual conformational changes of the binding process defines the physical site size n (M. Charlier and J.-C. Maurizot, unpublished results). This is the site that is determined in our analysis of the fluorescence data.

Results

Fluorescence Measurements. The intrinsic fluorescence of the *lac* repressor headpiece is strongly quenched upon complex formation with nucleic acids as well in the nonspecific as in the specific binding mode. This phenomenon can be used to determine the experimental binding density ν from

$$\nu = \frac{L_b}{P_0} = \frac{L_{tot}f_f - F}{(f_f - f_b)P_0} \quad (5)$$

with L_{tot} being the total headpiece concentration, F the measured fluorescence intensity, and f_f and f_b the fluorescence intensities per mole of headpiece in the free and the fully bound state, respectively.

Experimental binding isotherms were determined by adding small volumes of the concentrated headpiece stock solution to a DNA solution, working, thus, practically at constant DNA concentration P_0. To proceed in this way avoids a variation of the inner filter effect due to the absorption of the nucleic acid at the excitation wavelength. The parameter f_b was determined from the tangent at the origin of a plot F as a function of L_{tot} for a titration done in the low ionic strength binding buffer, assuming that initially all the headpiece is bound at this low ionic strength. This assumption has been independently shown to hold true by circular dichroism measurements (see below). The parameter f_f may be obtained either from measurements in the absence of DNA, adapting these values to that obtained in the presence of DNA taking into account the inner filter effect (Hélène et al., 1971), or from measurements in the presence of DNA, but at high ionic strength (we have used 1 M NaCl) assuming a complete dissociation of the complex at this ionic strength. Within experimental error both methods gave the same values, taking into account the slight enhancement (about 3%) of the headpiece fluorescence in 1 M NaCl as compared to the fluorescence in the binding buffer. The ratio f_b/f_f was always found in the range 0.36 ± 0.03 and seems to be independent of the nucleic acid base composition. Of course, the absolute values of f_b and f_f do not have a direct physical significance as they depend on the instrumental conditions (amplification, slit width, etc.).

Figure 1 shows same experimental binding isotherms as determined from eq 5 for different concentrations of NaCl. By far, the best fits of the experimental data by theoretical binding curves as deduced from eq 3 and 4 are those using n = 3 base pairs. Primarily, the isotherms at low ionic strength are very discriminating with respect to this parameter. Using n = 3, we are able to determine the degree of saturation of the DNA lattice $\theta = n\nu$ ($0 \le \theta \le 1$). Figure 1 shows that even at low ionic strength we reach only about 70% of the total lattice saturation under these experimental conditions. This is mainly due to the accumulation of gaps smaller than three residues in length between bound ligand molecules, making complete lattice saturation experimentally unobtainable

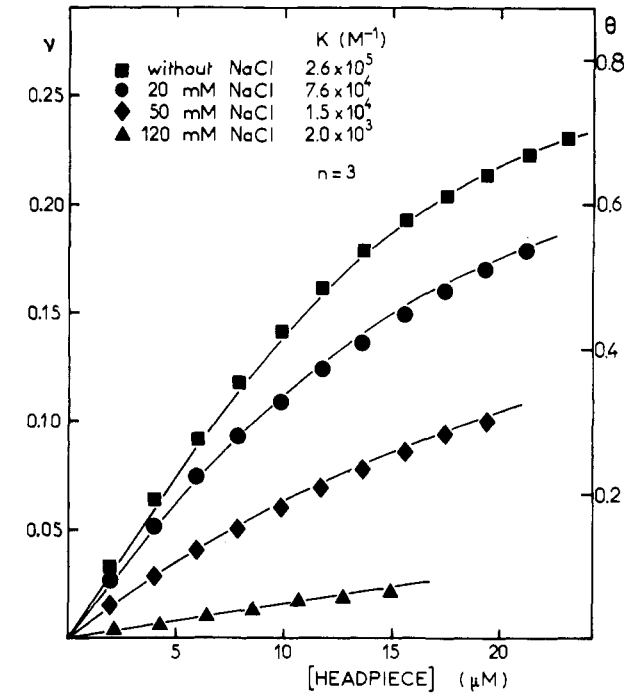

FIGURE 1: Experimental (■, ●, ♦, ▲) and theoretical (—) binding isotherms for interaction of headpiece with DNA as a function of total headpiece concentration at various NaCl concentrations, indicated in the figure, at pH 7.25. Other experimental conditions: 22 °C; concentration of DNA 6 × 10^{-5} M base pairs.

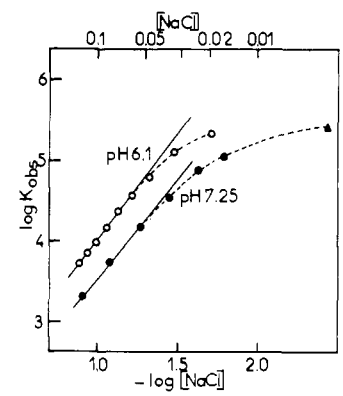

FIGURE 2: Dependence of log K_{obsd} on −log [NaCl] for interaction of headpiece with DNA at pH 6.1 (○) and 7.25 (●). All NaCl concentrations are corrected for the small amount of potassium (3.6 mM) in the solution. The point marked by a triangle (▲) represents the presence of potassium only (no NaCl in the solution). This correction does not influence the value of the final slope. Other experimental conditions are as in Figure 1.

(McGhee & von Hippel, 1974). The good agreement between experimental and theoretical values from an equation describing a noncooperative type of binding suggests that the nonspecific binding of the headpiece to DNA is in fact noncooperative.

Dependence of K_{obsd} on NaCl Concentration. Figure 2 shows the dependence of log K_{obsd} as a function of log [NaCl] for the interaction of the headpiece with DNA at two different pH values. The values at pH 7.25 are obtained with complete binding isotherms at six different NaCl concentrations. Another, more rapid procedure was used for the other values shown in Figures 2 and 3. Once the binding site size n is established, the salt dependence of the binding constant can be determined by a stepwise dissociation of the complex upon addition of small amounts of a concentrated salt solution (5 M NaCl). Introducing a slight correction of F for the dilution (never exceeding 3%) allows one to determine ν as before from

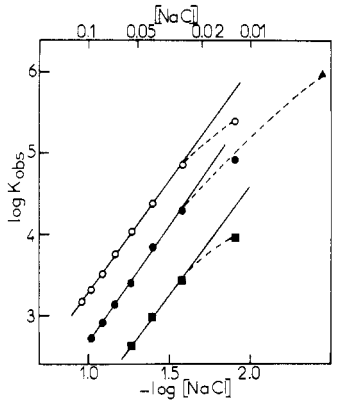

FIGURE 3: Dependence of log K_{obsd} on −log [NaCl] for interaction of headpiece with poly[d(G-C)] at pH 6.3 (○), 7.25 (●), and 8.2 (■). As in Figure 2, the NaCl concentration is corrected for the small amount of potassium. Other experimental conditions are as in Figure 1.

Table I: Equilibrium Parameters

	temp (°C)	pH	$-\partial \log K_{obsd} / \partial \log [NaCl]$	Z^a	log K at 1 M NaCl (extrapolated)
DNA	22	7.25	2.5 ± 0.3	2.8 ± 0.3	1.1 ± 0.4
DNA	22	6.1	2.6 ± 0.3	3.0 ± 0.3	1.4 ± 0.4
DNA	3.5	7.25	2.7 ± 0.3	3.1 ± 0.3	1.1 ± 0.4
poly[d(G-C)]	22	6.3	2.8 ± 0.3	3.2 ± 0.3	0.5 ± 0.4
poly[d(G-C)]	22	7.25	2.9 ± 0.3	3.3 ± 0.3	−0.2 ± 0.4
poly[d(G-C)]	22	8.2	2.7 ± 0.3	3.1 ± 0.3	−0.8 ± 0.4

[a] Assuming no anion release and $\psi = 0.88$.

eq 5. This value of ν was used to calculate K_{obsd} directly from a transformed version of eq 3. Figure 2 shows that the binding is stronger at the lower pH value over the whole ionic strength range. log K_{obsd} varies linearly with log NaCl for NaCl concentrations greater than 50 mM, whereas both plots are curved for smaller concentrations of salt. The slopes of the linear parts of the plots are nearly parallel (see Table I). At more basic pH values, the association constants are further diminished, and within the measurable range of association constants, the log–log plots are always curved and do not allow the determination of the final slope.

The observed curvature may be due to several reasons. Anion release from the protein upon binding to the DNA should lead to curvature, because the association constants for the protein–anion interaction are expected to be small, leading to an uncomplete saturation of the anion binding site(s) on the protein at lower salt concentrations. A conformational change of the protein with salt concentration might lead to a curvature too. We have shown earlier (Schnarr & Maurizot, 1982b) that the stability of the headpiece depends strongly on the salt concentration.

However, at least in part this curvature seems to be due to the nucleic acid base composition. Figure 3 shows the salt dependence of log K_{obsd} for the interaction with poly[d(G-C)] for three different pH values. The curvature is less pronounced, and all plots are linear below 25 mM NaCl. In the case of poly[d(A-T)], the curvature is at least as pronounced as with the natural DNA.

Table I summarizes the slopes of these plots, the number Z of ion pairs formed under the assumption of no anion release, and the extrapolated logarithms of the association constant at 1 M NaCl. In principle, these values would provide estimates of the nonelectrostatic contributions to the free energy of association. However, if pH and other environmental effects are involved, as is the case for the headpiece–DNA interaction,

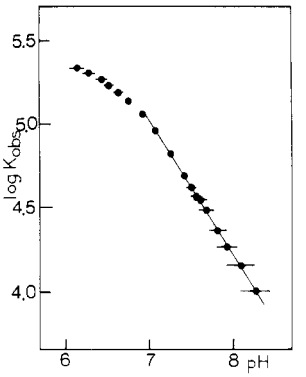

FIGURE 4: Dependence of log K_{obsd} on pH for interaction of headpiece with DNA in the presence of 20 mM NaCl. Other experimental conditions are as in Figure 1.

these values cannot be simply interpreted (de Haseth et al., 1977). The slopes of the log–log plots are practically independent of the pH value, an important finding that will be discussed below. All the measurements lead to an upper limit of three ion pairs formed, corresponding to the binding site size of three base pairs.

A comparison of the binding data for DNA and poly[d(G-C)] reveals that the headpiece binds about 4–6 times stronger to DNA for NaCl concentrations greater than 30 mM, that identical binding constants are observed at about 10 mM NaCl, and that the binding to poly[d(G-C)] is about 4 times stronger at very low ionic strength. This behavior might be due to a sequence-dependent counterion binding at low ionic strength, but we are not aware of previous results indicating such a phenomenon.

Dependence of K_{obsd} on pH. Figure 4 shows that the headpiece–DNA interaction depends strongly on the pH value. In the pH range 7.1–8.3, log K_{obsd} decreases linearly with pH; at pH values smaller than 7.0, the plot becomes curved, and at around pH 6.1, the association constant tends to be independent of pH. A prerequisite for these measurements was to study the pH dependence of the fluorescence of the isolated headpiece. It was shown to be independent of pH in the range 6.0–8.4. At more basic pH values, the fluorescence quantum yield decreases due to the onset of tyrosine deprotonation; at more acidic values, the quantum yield increases. This is in general not observed for tyrosine fluorescence (Alev-Behmoaras et al., 1979) and is probably due to the protonation of glutamic or aspartic side chains in proximity of one or several tyrosine residues. Ribeiro et al. (1981a) have reported shifts in the position of same tyrosine resonances in this pH range. Further, we have not seen any change in the circular dichroism spectra of the isolated headpiece in the pH range studied (5.2–9.0), suggesting pH independence of the headpiece structure in this pH range in agreement with Ribeiro et al. (1981a).

The extended linear zone in Figure 4 over the pH range 7.1–8.5, with a slope of −0.8 ± 0.2, is consistent with the behavior predicted by eq 2 and suggests strongly that the formation of a nonspecific headpiece–DNA complex requires the protonation of one titrating group of the headpiece above its normal pK. This is suggested further by the finding that the slopes of the log K_{obsd}–log [NaCl] plots (see Table I) are pH independent. A simple titration curve model (see Materials and Methods) cannot explain our data, because the slopes of the log–log plots should be pH dependent and, at basic pH, log K_{obsd} should get pH independent. Equation 2 predicts further that the pH value for which the slope is half of its alkaline value corresponds to the pK of the titrating group.

Table II: Effect of Temperature on DNA–Headpiece Interaction

temp (°C)	K_{obsd} (M^{-1})
3.5	1.20×10^5
9.7	1.17×10^5
14.0	1.15×10^5
18.6	8.91×10^4
22.0	7.59×10^4

We determine a pK value of 6.7 ± 0.3 from Figure 4. Taking into account that this group must be accessible at the surface of the protein, thus exhibiting a near-normal titration behavior, it should be either residue histidine-29 or the terminal α-NH_2 group. Both groups seem to fulfill the requirement of accessibility (Arndt et al., 1981; Ribeiro et al., 1981b). By NMR measurements, the pK of His-29 has been determined to be 7.0 in D_2O and 6.6 in H_2O (Ribeiro et al., 1981a; Wemmer et al., 1981), which is consistent when taking into account the deuterium effect or the pH electrode. The pK of the α-NH_2 group has been estimated to be at about 8.0 in D_2O as determined from a slight shift of a resonance attributed to residue methionine-1 (Ribeiro et al., 1981a). Correction of this value for the deuterium effect leads to a pK of about 7.6 in H_2O. Our value of 6.7 ± 0.3 agrees very well with that determined for histidine-29 but not with that determined for the α-NH_2 group. Nick et al. (1982) have reported that the histidine-29 C-4 proton resonance shifts downfield upon interaction with nonoperator DNA and that it is discernible at 7.22 ppm at the end of the titration with DNA. This chemical shift value is nearly identical with that reported by Ribeiro et al. (1981a) for the fully protonated form of histidine-29 (7.23 ppm), suggesting that the histidine residue becomes in fact protonated upon nonspecific complex formation.

Our result is also in agreement with that obtained by Scheek et al. (1983), who concluded from NMR experiments on the binding of the headpiece to a synthetic 14 base pair *lac* operator fragment that the large shifts from the histidine-29 C-2 and C-4 resonances result from protonation of this residue during the titration with the operator fragment.

Dependence of K_{obsd} on Temperature. The temperature dependence of K_{obsd} at fixed NaCl concentration and pH (20 mM NaCl, pH 7.25) is given in Table II. To determine the experimental binding density ν, we have used again eq 5. However, it must be taken into account that f_f and f_b depend on the temperature. We have first determined the temperature dependence of f_f over the temperature range used (3.5–22 °C). Decreasing the temperature from 22 to 3.5 °C increased the fluorescence quantum yield of the free headpiece by about 20%. Further, a complete binding curve was determined at 3.5 °C in low ionic strength binding buffer. This curve revealed that the ratio f_b/f_f is the same as that found at 22 °C. From the known temperature dependence of f_f and the known temperature-independent ratio f_b/f_f, the value of f_b may be calculated for each temperature. The temperature dependence may thus be determined on the same sample simply by variation of the temperature and determination of F. The data in Table II show that binding is slightly stronger at lower temperature under these experimental conditions. However, the van't Hoff plot of these data is nonlinear, as previously reported for the interaction of the entire *lac* repressor with nonoperator DNA (de Haseth et al., 1977).

Circular Dichroism Measurements. The binding of the headpiece on nonoperator DNA induces important changes of the nucleic acid circular dichroism. Figure 5 shows the important increase of the CD spectrum, as well as the difference spectrum. These changes are very similar to, if not identical with, those induced by the entire *lac* repressor upon binding an alternating polynucleotide (Maurizot et al., 1974) and natural nonoperator DNA (Butler et al., 1977; Culard & Maurizot, 1981). Butler et al. (1977) have suggested that these changes might be due to a transition of the DNA from the B to the A conformation. In fact, the observed difference spectrum in Figure 5 with a maximum at about 271 nm and a small shoulder at about 290 nm agrees qualitatively rather well with the difference spectrum for a B → A transition (Sprecher et al., 1979).

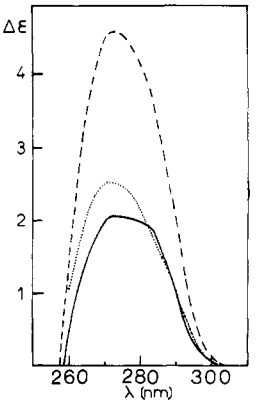

FIGURE 5: Circular dichroism spectra of DNA in the absence (—) and in the presence of the headpiece (– –) (0.34 headpiece per base pair) at a total DNA concentration of 5×10^{-4} M base pairs, as well as the corresponding difference spectrum (···). Other experimental conditions: 22 °C, pH 7.25.

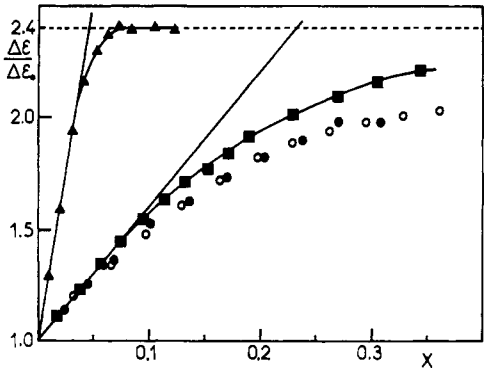

FIGURE 6: Relative increase of circular dichroism of DNA at 275 nm due to binding of headpiece for two different DNA concentrations as a function of total number of headpieces per base pair: (■) 5×10^{-4} M base pairs; (○ and ●) 6×10^{-5} M base pairs for two different headpiece preparations. For comparison, we have represented the results obtained for the *lac* repressor too (▲). Other experimental conditions are as in Figure 5.

Figure 6 shows the relative increase of the circular dichroism at 275 nm as a function of X, the total protein concentration per mole of base pairs, for the headpiece and the entire *lac* repressor in low ionic strength buffer. In the very initial part of the curves, the effect on the circular dichroism induced by the headpiece is independent of the total DNA concentration, indicating that even at the lower DNA concentration (mainly used for the fluorescence measurements), all the headpiece is bound for small X values. At higher X values, both curves are nonlinear. The observed curvature is of course first due to the fact that not all the added headpiece is bound but second due to the fact that the headpiece perturbs a greater number of base pairs (m) than its real physical size of n base pairs. If one assumes that the final perturbation of the circular dichroism of the DNA saturated with headpiece is the same as that with *lac* repressor ($\Delta\epsilon/\Delta\epsilon_0 = 2.4$ at 275 nm), a linear extrapolation of the slope at the origin leads to a perturbed

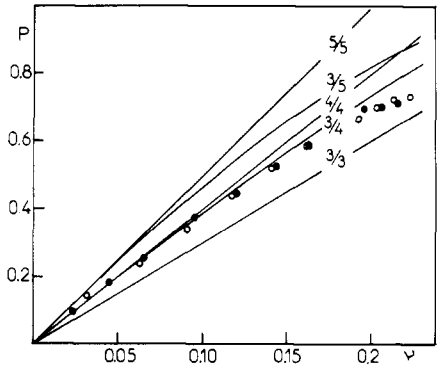

FIGURE 7: Normalized increase of circular dichroism [$P = (\Delta\epsilon - \Delta\epsilon_0)/(\Delta\epsilon_{max} - \Delta\epsilon_0)$] of DNA ($6 \times 10^{-5}$ M base pairs) at 275 nm as a function of number of *bound* headpieces per base pair for two different headpiece preparations, using $\Delta\epsilon_{max}/\Delta\epsilon_0 = 2.4$. The full lines are calculated according to eq 6 for the indicated parameters n/m.

site size of 4.3 ± 0.3 base pairs as compared to the physical site size of $n = 3$ base pairs determined from fluorescence measurements. If $m > n$ holds true, the observed perturbation of the circular dichroism should depend nonlinearly on the binding density ν, because at higher binding densities headpiece molecules will bind to base pairs already perturbed by adjacent ligands. Figure 7 shows that the increase of the circular dichroism (normalized to vary between 0 and 1) as a function of ν (determined in parallel by fluorescence measurements) is in fact nonlinear with a slope at $\nu = 0.2$ of only half of that observed at low binding density. A similar phenomenon has been observed for the interaction of the entire *lac* repressor with nonoperator DNA. To analyze those data quantitatively, M. Charlier and J.-C. Maurizot (unpublished results) have developed the following equation:

$$P(m,n,\nu) = \nu\left(n + \frac{1 - n\nu}{\nu}\left[1 - \left(\frac{1 - n\nu}{1 - (n-1)\nu}\right)^{m-n}\right]\right) \quad (6)$$

Figure 7 shows that our experimental values are best fitted by using $n = 3$ and $m = 4$, in agreement with the direct extrapolation ($m = 4.3 \pm 0.5$).

Discussion

The fact that some quenching of the fluorescence of the headpiece occurs when it is bound on the nucleic acid is not surprising. It has been shown with model peptides containing tyrosine (Hélène & Maurizot, 1981; Hélène & Lancelot, 1982) that their binding on nucleic acids is accompanied by a tyrosine fluorescence quenching. Care must be taken in the interpretation of such a result. It does not necessary imply that some tyrosine residue(s) of the headpiece is (are) intercalated or stacked with the bases of the nucleic acid. Other types of mechanism could explain tyrosine quenching: proton transfer in the excited state (Alev-Behmoaras et al., 1979) or energy transfer. It has been calculated (Montenay-Garestier, 1975) that the critical Förster distance for the singlet–singlet energy transfer of tyrosine to the nucleic acid base is about 20 Å. Taking into account the small size of the headpiece, it is very likely that some of the four tyrosine residues are located at a distance of the DNA that allows such a transfer.

Small-angle neutron and X-ray scattering experiments on the core and on the *lac* repressor have shown that the headpieces are situated in pairs at either end of an elongated molecule (Charlier et al., 1980, 1981; McKay et al., 1982). This fact has led to the proposal that the *lac* repressor binds to nucleic acids via two headpieces. Other data supporting a model in which only two protomers constitute the DNA binding site have been provided by the use of chimeric β-galactosidase repressor (Kania & Brown, 1976; Kania & Müller-Hill, 1977) and hybrid repressor-core tetramers (Geisler & Weber, 1976). Furthermore, it has been shown by Culard et al. (1982) that two headpieces bind specifically on the *lac* operator sequence. On the basis of the crystallography results on Cro and λ repressors (Anderson et al., 1981; Pabo & Lewis, 1982) and CAP protein (McKay & Steitz, 1981), it has been recently proposed that the *lac* repressor binds to the *lac* operator by two headpieces in which two α-helical segments make contacts with the major groove of DNA (Sauer et al., 1982; Weber et al., 1982; Matthews et al., 1982). On the other hand, using hybrid tetramers containing different ratios of core to native subunits, Dunaway & Matthew (1980) have proposed that two subunits make major contacts with the operator sequence and two other minor contacts. In the model deduced from these experiments (Dunaway et al., 1980), the description of the nonspecific binding is less explicit and proposes that each NH_2 terminus in the tetrameric structure can bind to nonspecific DNA independently. It is therefore interesting to make a comparison between the binding parameters that we have established for the headpiece in this study and those previously found for the binding of the *lac* repressor.

Among the parameters determined in this study, one of the most interesting is the number of protonated groups during the interaction. We found that this number is one, probably histidine-29, whereas it has been found by de Haseth et al. (1977) that this number is two for the *lac* repressor. This strongly favors the hypothesis that two headpieces are involved in the binding of the *lac* repressor to DNA even in the nonspecific binding mode. The protonation of a residue above its normal pK may be due to the fact that it participates directly in the binding process on the DNA by an electrostatic interaction. Another explanation for such a phenomenon could be that the protonation induces a conformational change of the headpiece necessary for the binding to the nucleic acid. This seems rather unlikely in view of the absence of any change in the headpiece circular dichroism spectrum in the peptide chromophore wavelength range with pH.

The number of phosphate groups involved in the nonspecific interaction between *lac* repressor and DNA is between 10 and 12, whereas the number found for the headpiece is 3. It must be pointed out that our experiments are performed with the short headpiece ending at residue 51. One may suppose that this arginine residue bearing the negatively charged carboxylate terminal group is not available for electrostatic interaction. Such a phenomenon has been shown in studies using model peptides (Hélène & Maurizot, 1981; Hélène & Lancelot, 1982). Lysine-59 might also participate in the *lac* repressor–DNA interaction and could explain why the number of electrostatic interactions found for the headpiece is not half of that found for the *lac* repressor. The possibility that one of these or both residues are involved in *lac* repressor binding is reinforced if one considers that the long headpiece binds more strongly to nonoperator DNA (Jovin et al., 1977) and to operator DNA (Ogata & Gilbert, 1979) than to the short headpiece.

The latter contains six basic amino acids plus histidine-29 and the α-NH_2 terminal group, which can bear positive charges. Clearly, only a part of these positive groups is involved in electrostatic interactions. It would be of interest to test which of these groups are not involved in the interaction. For example, Miller et al. (1979), using suppression of non-

sense mutations, have shown that the lysine in position 2 can be replaced by several uncharged amino acids without alteration of the biological activity of the repressor.

The physical site size as determined from fluorescence analysis is three base pairs long. This value is in good agreement with the CD result that indicates that about four base pairs are perturbed (from a CD point of view) and with the analysis of melting profiles (Durand et al., 1983) shows that four base pairs are protected against thermal denaturation by the binding of one headpiece. Recently, the nonspecific interaction of the λ phage Cro protein with DNA has been studied in detail by Boschelli (1982). The author finds a physical site size varying between about four and eight base pairs (depending on ionic strength) per Cro *dimer*. If one takes into account that the isolated headpiece interacts with DNA very probably as a monomer, our results compare quite favorably with those found for the Cro repressor. Large discrepancies exist in the value of the site size reported for the *lac* repressor. These values range from 11 to 33 base pairs. In any case, the value of the site size that we found for the headpiece is much smaller than that for the *lac* repressor and even smaller than half of this value. Even if two headpieces of the *lac* repressor interact with the DNA, this is not surprising since one may expect that in the *lac* repressor the two headpieces that interact with the DNA are separated by the core and not just adjacent.

Measurements of the binding constants of *lac* repressor to DNA have been reported. They have been obtained by using a sedimentation velocity technique with λ DNA (Revzin & von Hippel, 1977), DNA chromatography (de Haseth et al., 1977), and a difference boundary sedimentation velocity technique on T7 DNA (Lohman et al., 1980). It is difficult to make a direct comparison between the values found for the *lac* repressor and our values for the headpiece since the binding of *lac* repressor was analyzed in buffers containing more than 0.1 M NaCl whereas our measurements were mainly performed at ionic strengths lower than 0.1 M. An extrapolation from the data on the *lac* repressor indicates that at 0.1 M NaCl the binding constant should be of the order to 10^7 M^{-1}. At the same ionic strength, our data show that the binding constant of the headpiece is about 10^3 M^{-1}. This value is about the square root of that of the *lac* repressor as would be expected if the *lac* repressor interacts by two headpieces with the DNA. However, care must be taken before definite conclusions are deduced from this observation. As pointed out by Ogata & Gilbert (1979), the assumption that the affinity of a dimer corresponds to the square of the affinity of a monomer is only approximately true since it does not take into account the entropic effect of the process. Furthermore, as we have mentioned earlier, the number of electrostatic interactions involved in the binding of the short headpiece is not half of that observed for the *lac* repressor. If for one ionic strength $K_{rep} = K_{HP}^2$ holds true, clearly this will not be exact for other ionic strengths.

The comparison between the parameters we have determined for the short headpiece and those obtained for the *lac* repressor clearly exclude that one headpiece constitutes the nonspecific binding site of DNA in *lac* repressor. It strongly favors the idea that two headpieces are involved in such a binding and suggests that residues arginine-51 and/or lysine-59 participate in the electrostatic interaction of *lac* repressor with DNA.

What is the conformational change of the DNA induced by the binding of the headpiece? As we have noticed, the CD change is very similar in shape to that observed when DNA undergoes the B → A transition in alcoholic medium. However, the site size is very short, three base pairs, and the length perturbed on the DNA even if it is larger does not extend by more than one or two base pairs over the steric site. As the B → A transition is cooperative, it would be surprising that such a short segment could be in the A form. Selsing et al. (1979) have generated a model for the junction of contiguous DNA segments having A-DNA and B-DNA conformation. The junction needs one base pair and the two neighboring internucleotide linkages in a particular non-A and non-B geometry. A region in the A form, due to the binding of the headpiece, would need two junctions, and the site size seems too small to account for such a hypothesis. Another argument against the B → A transition comes from the intensity of the effect. In a B → A transition, the intensity of the positive band of the CD signal is multipled by a factor of about 4. The CD increase that we observe would correspond to only two base pairs in the A form per bound headpiece. It should be pointed out that the circular dichroism of nucleic acids is very sensitive to many parameters (number of residues per turn, relative orientation of the base plates, "propeller twist" within one base pair, etc.), and additional methods are clearly needed to characterize the induced conformation. Trials in our laboratory to obtain an oriented headpiece–nucleic acid film for a study by infrared linear dichroism were not successful.

Acknowledgments

We are grateful to A. Gervais for help in preparing the *lac* repressor and its fragments. We thank M. Charlier, F. Culard, and C. Hélène for helpful discussions.

Registry No. Sodium chloride, 7647-14-5.

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