^{31}P Nuclear Magnetic Resonance Spectra and Dissociation Constants of *lac* Repressor Headpiece·Duplex Operator Complexes: The Importance of Phosphate Ester Backbone Flexibility in Protein·DNA Recognition†

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Received November 18, 1992; Revised Manuscript Received April 16, 1993

ABSTRACT: An alkaline phosphatase assay was used to determine the dissociation constants (K_D) of the *lac* repressor N-terminal 56 amino acid fragment of the wild type and of a Y7I mutant complexed to 22 base pair (bp) wild-type and mutant symmetrical operator sequences. K_D's in 0.35 M monovalent salt ranged from 5.4×10^{-8} M for the wild-type repressor·wild-type operator complex to $\gtrsim 1 \times 10^{-6}$ M for the wild-type repressor·nonspecific DNA complex. Mutant operators O2 (G5 → A5 and C16 → T16) and O4 (G5 → C5 and C16 → G16) bind nearly as tightly as the wild-type headpiece, while mutant O3 (A8 → T8 and T13 → A13) binds over 5-fold poorer. Operators O1, O2, and O4 bind ca. 10-fold poorer to the Y7I mutant headpiece. Operator O3 binds 2-fold poorer to the mutant headpiece. The temperature and salt dependence on the dissociation constants of wild-type headpiece binding to 22-bp operator support the conclusion that the headpiece contains the major DNA recognition portion of the protein and that electrostatics plays as important a role in the binding of operator to headpiece as it does in the whole repressor. The ^{31}P NMR spectra of shortened 14-bp wild-type and mutant symmetrical operators bound to the N-terminal 56-residue headpiece of the Y7I mutant repressor were compared to the spectra of the same operator bound to the wild-type repressor headpiece. These results are consistent with a recent proposal [Karslake, C., Botuyan, M. V., & Gorenstein, D. G. (1992) *Biochemistry 31*, 1849–1858] that specific, tight-binding operator·protein complexes retain the inherent phosphate ester conformational flexibility of the operator itself, whereas the phosphate esters are conformationally restricted in the weak-binding operator-protein complexes. This retention of backbone torsional freedom in tight complexes is entropically favorable and provides a mechanism for protein discrimination of different operator binding sites.

How do proteins recognize DNA? Most attention toward understanding the binding specificity between amino acid sequences and DNA sequences has centered on hydrogen bonding to the acceptor/donor groups on the Watson–Crick base pairs in the major groove [cf. Landschulz et al. (1988)]. At present we do not understand this "second genetic code" of protein·DNA recognition. Perhaps one reason for the inability to dissect the basis for this specificity is the emphasis on base-pair interactions alone. Localized, sequence-specific conformational variations in DNA are quite likely another important component of a protein's recognition of specific sites on the DNA (Landschulz et al., 1988; Matthews, 1988).

Remarkably, in every high-resolution X-ray crystal structure of a protein·DNA complex, the majority of the contacts are to the phosphates (Beamer & Pabo, 1992; Jordan & Pabo, 1988; Otwinowski et al., 1988; Somers & Phillips, 1992; Wolberger et al., 1988). Indeed, the crystal structure of the *trp* repressor demonstrated that every one of the direct protein contacts was mediated through interactions with the phosphate backbone (Otwinowski et al., 1988). While some controversy exists as to the correctness of this complex (Marmorstein et al., 1991; Staacke et al., 1990), more recent NMR results confirm the specificity of this operator complex (Haran et al., 1992; O. Jardetzky, Abstracts, International Biological NMR Meeting, Jerusalem, 1992). Ionic interactions involving the phosphate backbone have been implicated as important factors in the recognition of the *lac* operator as well (deHaseth et al., 1977a,b; Karslake et al., 1990). It is not known whether any of these ionic interactions provide a specific recognition mechanism for these repressors.

The *lac* repressor system is ideal for studying DNA·protein interactions by NMR (Buck et al., 1978, 1980, 1983; Hogan et al., 1981; Nick et al., 1982; Scheek et al., 1983; Wade-Jardetzky et al., 1979; Wemmer & Kallenbach, 1983; Zuiderweg et al., 1985). It is possible to duplicate the basic *lac* operator·*lac* repressor protein interaction by using the smaller *lac* repressor headpiece N-terminal domain fragment (Adler et al., 1972; Buck et al., 1978; Wade-Jardetzky et al., 1979; Wemmer & Kallenbach, 1983; Zuiderweg et al., 1985). Kaptein and co-workers (Boelens et al., 1987; Zuiderweg et al., 1985) have assigned many of the ^{1}H signals of the *lac* repressor headpiece as well as those of the headpiece·operator fragment complexes by 2D NMR methods (Wuthrich, 1986). The ^{1}H spectrum of the Tyr 7 to Ile mutant *lac* repressor headpiece has also been assigned (Karslake et al., 1991; Wisniowski et al., 1992). NOESY distance-restrained molecular dynamics studies of the repressor headpiece bound to *lac* operator DNA fragments have shown that a recognition α-helix binds within the major groove of the operator DNA.

As described in this article, by comparing changes in binding affinity and changes in the ^{31}P spectra of small operator fragments upon binding wild-type and mutant *lac* repressor headpieces, we support our earlier suggestion (Karslake et al., 1991, 1992; Wisniowski et al., 1992) that the conformation and, in particular, the flexibility of the phosphates as well as

† Supported by the NIH (AI27744), the Purdue University Biochemical Magnetic Resonance Laboratory, which is supported by the NIH designated AIDS Research Center at Purdue (AI727713), and the NSF Biological Facilities Center on Biomolecular NMR, Structure and Design at Purdue (Grants BBS 8614177 and 8714258 from the Division of Biological Instrumentation).

0006-2960/93/0432-6863$04.00/0

O1

$5' - G_{p0}A_{p1}A_{p2}T_{p3}[T_{p4}G_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}C_{p16}A]_{p17}A_{p18}T_{p19}T_{p20}C_{p21}$

O2

$5' - G_{p0}A_{p1}A_{p2}T_{p3}[T_{p4}\mathbf{A}_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}\mathbf{T}_{p16}A]_{p17}A_{p18}T_{p19}T_{p20}C_{p21}$

O3

$5' - G_{p0}A_{p1}A_{p2}T_{p3}[T_{p4}G_{p5}T_{p6}G_{p7}T_{p8}G_{p9}C_{p10}G_{p11}C_{p12}\mathbf{A}_{p13}C_{p14}A_{p15}C_{p16}A]_{p17}A_{p18}T_{p19}T_{p20}C_{p21}$

O4

$5' - G_{p0}A_{p1}A_{p2}T_{p3}[T_{p4}\mathbf{C}_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}\mathbf{G}_{p16}A]_{p17}A_{p18}T_{p19}T_{p20}C_{p21}$

NS

$5' - C_{p0}C_{p1}C_{p2}A_{p3}G_{p4}C_{p5}A_{p6}A_{p7}C_{p8}C_{p9}T_{p10}A_{p11}G_{p12}G_{p13}T_{p14}T_{p15}G_{p16}C_{p17}T_{p18}G_{p19}G_{p20}G_{p21}$

FIGURE 1: Symmetric 22-bp wild-type operator O1 and mutant operators O2–O4. NS is the nonspecific sequence used in the binding affinity assay. The 14-bp fragments (O1′–O4′) are indicated in brackets. Numbering of the residues is shown.

the backbone of the headpiece may be important components of protein·DNA recognition.

EXPERIMENTAL SECTION

Synthesis of Operators. The HPLC-purified self-complementary *lac* wild-type and mutant 22-bp symmetric operators (1 OD each) (O1–O4) were purchased (Operon Technologies, Alameda, CA) for the alkaline phosphatase assay. The purity of the 22-mer operators was confirmed by denaturing gel electrophoresis. A single band was observed on a 25-cm 12% polyacrylamide gel in 20 M formamide for each of the operators. The sequence of the operators and numbering scheme for the phosphates are shown in Figure 1.

The 14-bp fragments of the four operators, wild-type $d(TGTGAGCGCTCACA)_2$ (O1′), as well as three mutants, $d(TATGAGCGCTCATA)_2$ (O2′), $d(TGTGTGCGCACACA)_2$ (O3′), and $d(TCTGAGCGCTCAGA)_2$ (O4′)—complementary sites of mutation in the palindromic operators are in boldface type—were synthesized on a 10 μmol scale and purified as previously described (Schroeder et al., 1987). The 14-mer operators are numbered beginning at base 4 and extending through base 17 (shown in brackets in Figure 1).

Wild-Type and Y7I Mutant lac Repressor Headpiece. The tyrosine 7 to isoleucine mutant holorepressor (Y7I) was constructed by oligonucleotide-directed site-specific mutagenesis on the plasmid pHIQ6, as previously described (Karslake et al., 1990; Wisniowski et al., 1992).

The N-terminal 56-residue headpieces of both wild-type and Y7I repressors were prepared following the procedure of Arndt et al. (1981), as previously described for the wild-type protein (Karslake et al., 1990), with the additional quenching of the α-chymotrypsin (Worthington) digest with a 4 molar equivalent excess of turkey ovomucoid trypsin inhibitor (Sigma). For several preparations, the headpiece was further purified by reversed-phase HPLC on a semipreparative C_{18} column (Vydac) with a linear gradient of acetonitrile/water with 0.1% trifluoroacetic acid (flow rate 1 mL/min, 0–60% acetonitrile over a period of 60 min). After HPLC, the headpiece fraction was concentrated by lyophilization, denatured with 8 M urea (6 h, 22 °C incubation), and renatured back to its native state by 5-fold dilution with a 0.03 M potassium phosphate buffer solution. Urea was removed by extensively dialyzing the sample against the same phosphate buffer. Yields of 7–13% of the N-terminal 56 amino acid fragment of the mutant and native repressors were obtained from 200 mg of the tetrameric headpiece.

The concentrations of native and mutant headpiece fragments were determined by UV using ϵ_{280} = 0.888 and 0.666 OD/mg, respectively. The wild-type and mutant headpieces were characterized by amino acid analysis, bio-ion^{252} Cf desorption mass spectroscopy, and 2D NMR (Karslake et al., 1991; Wisniowski et al., 1992).

^{31}P NMR samples were prepared by dissolving 4–6 mg of the lyophilized DNA in 0.4 mL of a 0.03 M potassium phosphate buffer solution containing 0.1 mg/mL sodium azide in D_2O. The ionic strength of the surrounding media is important for proper protein·DNA recognition. In order to provide the correct ionic strength for the phosphorus titration, solutions of both 14-mer operators and headpieces were separately centrifuged in Sephadex G-10 (Pharmacia) spun columns to ensure constant buffer conditions (Maniatis et al., 1982). The spun columns had been previously equilibrated to the correct ionic strength with at least 10 passes of the 0.03 M potassium phosphate buffer in D_2O, pH* (uncorrected pH meter reading) 7.4, prior to the addition of either the protein or the oligonucleotide. Both the headpiece and DNA concentrations were measured by UV spectroscopy. Amounts of the 14-mers were determined spectrophotometrically using the relationship of 20 OD units per mg of DNA at 260 nm. Typical initial concentrations of the headpiece and the DNA were 2–3 and 1–1.5 mM, respectively. At the end of the titration, these concentrations had been reduced by one-half, with a final reaction volume of 0.8 mL.

^{31}P NMR Spectra. The ^{31}P NMR spectra were run on a Varian XL-200A spectrometer at ambient temperature (ca. 25 °C) operating at 81.1 MHz. A sweep width of 172 Hz, acquisition time of 2.98 s, block size of 1K zero-filled to 16K, and a pulse width of 7 ms were used for the 1D spectra. Spectra were resolution enhanced using a combination of positive exponential and Gaussian apodization functions. Typical values were 0.1–0.2 resolution enhancement and 0.5–0.6 line-broadening apodization function values. The values were adjusted in accordance with the signal to noise ratio in the ^{31}P NMR spectrum. The number of acquisitions for each spectrum was typically between 2000 and 3000. The ^{31}P resonances were referenced to an external sample of trimethyl

phosphate (TMP) at 0.0 ppm, which is 3.53 ppm downfield of 85% phosphoric acid.

End-Labeling of 22-mers. Quantities (1–2 μg) of the various operators were 5′-^{32}P-labeled (Maxam & Gilbert, 1977) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP with a specific activity of 3000 Ci/mM (Amersham). Labeled oligonucleotides were separated from the [γ-^{32}P]ATP with a G50-80 Sephadex column equilibrated with 1× TE buffer.

The labeled fractions (3–4 mL) were pooled and lyophilized to dryness. The dried, labeled operators were then brought up in 100–200 μL of 0.2 M KCl solution. Aliquots (3 × 1 μL) were removed and the counts per minute determined with a Packard 1600A liquid scintillation counter. The average cpm was then used to approximate the labeled DNA concentration, assuming 20 000 cpm/μL equals 0.15 ng of DNA. The sample was then heated for 2 min at 90 °C and cooled slowly to anneal the DNA to duplex form. Relative amounts of double- and single-stranded DNA were determined by nondenaturing gel electrophoresis on a 25-cm 12% polyacrylamide gel (only duplex was observed at operator assay concentrations). The amount of radioactive DNA in the polyacrylamide gel bands was determined using a Pdi Quantity One densitometer. The annealing efficiency was usually 90–95%. This procedure yielded solution concentrations of 300–500 nM labeled duplex.

Alkaline Phosphatase Assay. Following the procedure reported for the *trp* repressor assay (Marmorstein et al., 1991), a 200-μL reaction mixture was prepared containing 20 nM DNA, 500 nM *lac* repressor headpiece, 0.2 M KCl, 10 mM TE (pH 7.4), and 20–30 μg/mL bovine serum albumin. Under comparable conditions (1 μM headpiece), the headpiece still had ordered secondary structure as revealed by circular dichroism experiments. After 30 min, two 20-μL aliquots were removed (for zero-time cpm values) and added to 100 μL of 0.15 M KP_i, pH 7.0, which serves to quench alkaline phosphatase activity. Longer incubation times of up to 3 h did not alter the results. Approximately 5–10 μL (1 unit of enzyme activity per μL) of alkaline phosphatase (EC 3.1.3.1; Boehringer Mannheim Biochemicals, Indianapolis, IN) in 30 mM TE, 3 M NaCl, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ (pH ~7.6) was added to the reaction mixture to start the digestion of the labeled DNA. When the enzyme was dialyzed against the reaction buffer, it effected higher rates of dephosphorylation (higher k_i and k_i'). However, the ratio k_i/k_i' was essentially the same as when the enzyme was not dialyzed. Subsequent experiments were performed using the enzyme in its storage (original) buffer. Aliquots (20 μL) were removed as a function of time and added to 100 μL of 0.15 M KP_i, pH 7.0. Typically, nine time points including the zero-time values were taken.

Once the time course was complete, 25–50 μL of a 20 mg/mL stock solution of t-RNA and 1 mL of 5% TCA were added to each quenched reaction mixture to precipitate the DNA·protein complex. The precipitated samples were then filtered through 0.45 μM, (pore size, 25 mm diameter) nitrocellulose filters (Millipore Corp., Bedford, MA). The reaction tube was washed with an additional 1 mL of 5% TCA and filtered through the same nitrocellulose membrane. The filters were dried for 1 h and the retained cpm measured. The slope of the line formed in a plot of ln (cpm/cpm_0) vs time was determined by a linear least-squares fit of the data. An example of a plot of ln (cpm/cpm_0) vs time for the wild-type headpiece·DNA complexes is shown in Figure 2. Reproducibility in measuring the rate constants is generally better than ±20%.

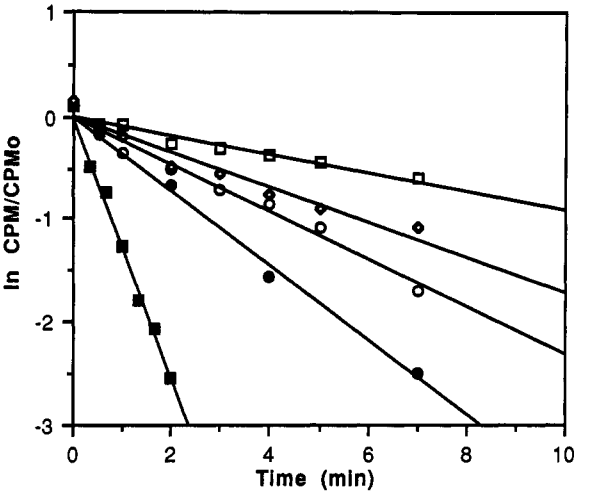

FIGURE 2: Plot of ln (cpm/cpm_0) vs time for the wild-type headpiece·DNA complexes: O1 operator, no HP (■); O1 operator plus HP (□); O2 operator plus HP (○); O3 operator plus HP (●); O4 operator plus HP (◇). Duplex DNA concentration was 20 nM and headpiece concentration was 500 nM under standard assay conditions.

In the absence of repressor,

$$\text{O-5′-}^{32}\text{P} \xrightarrow{k_i} \text{O} + {}^{32}\text{P}_i$$

where k_i is the rate constant for dephosphorylation in the presence of the enzyme, O is the operator, 5′-^{32}P-O is the 5′-labeled operator, and $^{32}P_i$ is liberated inorganic phosphate. In the presence of repressor,

$$\begin{array}{l} \text{O-5′-}^{32}\text{P} + \text{R} \overset{K_D}{\rightleftharpoons} \text{O-5′-}^{32}\text{P·R} \\ \quad k_i' \downarrow \\ \text{O} + {}^{32}\text{P}_i \end{array}$$

where R is the repressor, K_D is the equilibrium dissociation constant, and k_i' is the rate of dephosphorylation with the competing equilibrium present. The repressor acts to inhibit the alkaline phosphatase, and therefore $k_i > k_i'$.

In the presence of excess repressor,

$$[\text{R}] = [\text{R}]_0 - [\text{O-5′-}^{32}\text{P·R}] - [\text{O·R}] \cong [\text{R}]_0$$

In this case,

$$k_i' = k_i/(1 + [\text{R}]/K_D)$$

or, solving for K_D,

$$K_D = [\text{R}]/(k_i/k_i' - 1)$$

Thus, the rate at a particular alkaline phosphatase concentration is determined from the slope of the plot of ln (cpm/cpm_0) vs time for the operator with and without repressor present and is used to calculate the K_D of the complex (Marmorstein et al., 1991).

RESULTS

The alkaline phosphatase assay was first described by Marmorstein et al. (1991) for the determination of dissociation constants for the *trp* repressor/operator system. The assay is based on the decrease in the rate of dephosphorylation of 5′-^{32}P-labeled DNA by the enzyme alkaline phosphatase due to the presence of a DNA binding protein. In order for the

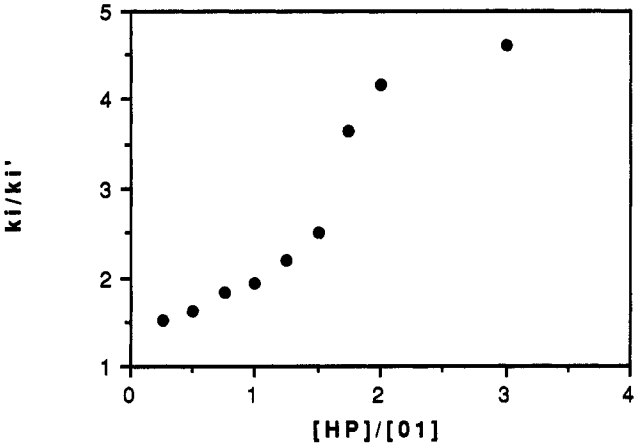

FIGURE 3: Plot of k_i/k_i' vs ratio of wild-type headpiece [HP] to 22-bp O1 operator [O1] concentrations. The DNA concentration was 1.0×10^{-7} M under standard assay conditions.

Table I: Dissociation Constants (M) of Native and Mutant *lac* Repressor Headpiece-Operator Complexes

	repressor headpiece	
operator[a]	wild type	Y7I
O1	$(5.4 \pm 1.1) \times 10^{-8}$	$(4.5 \pm 1.8) \times 10^{-7}$
O2	$(7.0 \pm 0.7) \times 10^{-8}$	$(5.7 \pm 0.7) \times 10^{-7}$
O3	$(2.6 \pm 1.1) \times 10^{-7}$	$(4.7 \pm 1.1) \times 10^{-7}$
O4	$(6.4 \pm 0.5) \times 10^{-8}$	$(6.7 \pm 0.6) \times 10^{-7}$
NS	$>(1.1 \pm 0.6) \times 10^{-6}$	

[a] The sequences of operators O1–O4 and NS are given in Figure 1. All values are the mean of 3–5 assays with units of moles/liter.

assay to work, the protein binding elements of the operator must be near the 5′ end of the sequence, so that they inhibit the enzymatic activity of the alkaline phosphatase.

Both the palindromic wild-type (O1; see Figure 1) 22-bp *lac* operator and mutant operators with mutations in the TGTGA binding elements were used in the assay with both the wild-type *lac* repressor headpiece and the Y7I mutant headpiece. Both longer (24 bp) and shorter (20 bp) fragments were not as effective (data not shown). At low salt concentrations (0–0.09 M), a 2:1 complex is formed for the 14-bp operators, as demonstrated by ^{31}P shift titration curves [cf. Karslake et al. (1990)]. For the longer 22-bp operators, at a salt concentration of 0.35 M, the headpiece also forms a 2:1 complex as shown by the break in the plot of k_i/k_i' vs [HP]/[O1] (Figure 3). Because of the limited precision of the measured rate constant data, it has not been possible to reliably determine the degree of cooperativity in the association of both headpiece molecules to the operator. All of the K_D's reported in Table I are at a 0.35 M monovalent cation concentration and assume no interaction between the binding sites.

Temperature Dependence of the Dissociation Constants. Analysis of the temperature dependence of K_D can provide information about the enthalpic and entropic contributions to the $\Delta G°$ of a protein/DNA interaction (Ha et al., 1989; Record et al., 1991). The K_D's determined by the alkaline phosphatase assay for wild-type repressor/O1 operator binding from 0 to 30 °C are listed in Table II and plotted in Figure 4A. The temperature dependence on the *lac* headpiece bound to a 22-bp operator shows behavior similar to that observed by Ha et al. (1989) for whole *lac* repressor binding to a 20-bp symmetric operator contained within a plasmid.

Normally, a van't Hoff analysis of the temperature dependence of the dissociation constant yields enthalpy and

Table II: Dissociation Constants (M) of Wild-Type·Wild-Type *lac* Repressor Headpiece-Operator Complexes as a Function of Temperature

temperature (°C)	K_D (M × 10^7)[a]
0	10.1 (±1.3)
10	7.0 (±2.2)
16	2.4 (±0.3)
22	0.54 (±0.11)
26	1.8 (±0.2)
30	5.6 (±0.8)

[a] All values are the mean of three assays.

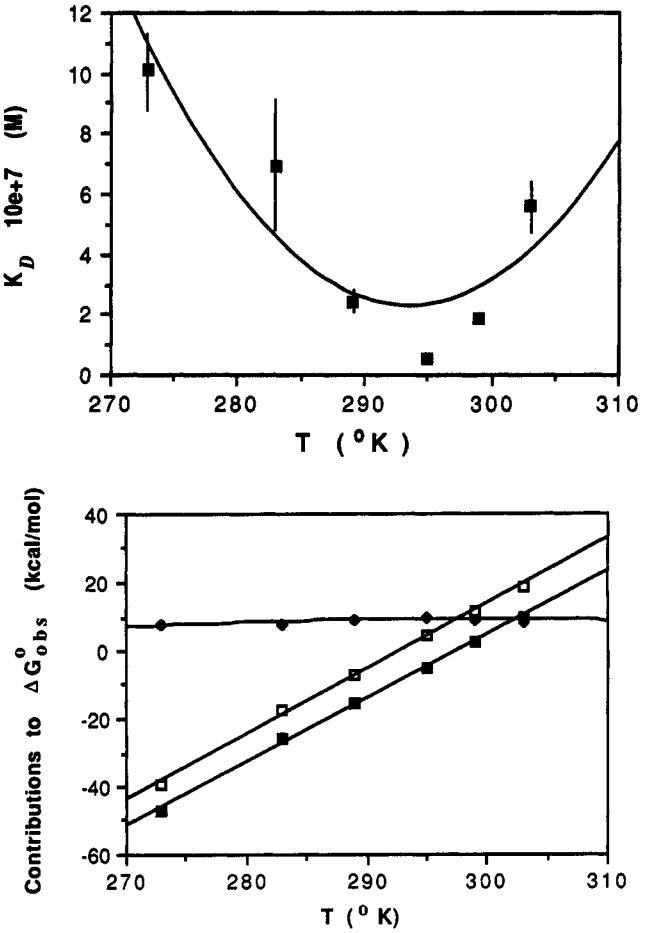

FIGURE 4: (A, top) Experimental K_D (■) vs temperature. Error bars are shown. (B, bottom) Thermodynamics of the interaction of the *lac* repressor headpiece with 22-bp operator O1. $\Delta G°_{obs}$ (◆), $\Delta H°_{obs}$ (□), and $\Delta S°_{obs}$ (■) are plotted vs temperature. Equations 1–4 were used to generate a theoretical fit to the data.

entropy changes which are temperature independent. However, the data for both the whole repressor (Ha et al., 1989) and the headpiece (Figure 4) are clearly nonlinear, and the simple van'Hoff analysis is inapplicable. Thus, Record and co-workers (Ha et al., 1989) observed a minimum in K_D at around 20 °C and larger values at higher and lower temperatures. This has been explained in terms of a large temperature-dependent heat capacity change with resultant entropy/enthalpy compensation (Record et al., 1991). For *lac* repressor protein/DNA interactions, both $\Delta H°_{obs}$ and $\Delta S°_{obs}$ decrease with increasing temperature with nearly the same slope, so that $\Delta G°_{obs}$ remains fairly constant. At low temperature the main driving force of complexation is entropic, while at higher temperatures enthalpic contributions dominate.

We have applied similar analysis to the headpiece interaction with operator. Following Record and co-workers, the data in

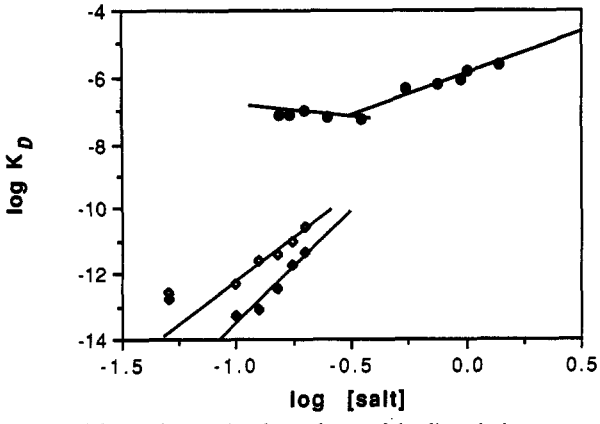

FIGURE 5: Monovalent cation dependence of the dissociation constants for the *lac* repressor protein [Winter and von Hippel (1981), ◆, ◇] and the headpiece (this study, ●). $MgCl_2$ and $ZnCl_2$ were also present in the reaction mixture at concentrations of 0.50 and 0.05 mM, respectively, for the headpiece study.

Table II have been analyzed using eqs 1–4:

$$\ln K_D = \left(\frac{\Delta C^\circ_{P,obs}}{R}\right)\left[\left(\frac{T_H}{T}\right) - \ln\left(\frac{T_S}{T}\right) - 1\right] \quad (1)$$

where $\Delta C^\circ_{P,obs}$ is the standard heat capacity change of the binding, T_S is the temperature where $\Delta S^\circ_{obs} = 0$, and T_H is the temperature where $\Delta H^\circ_{obs} = 0$. Using eqs 2–4,

$$\Delta G^\circ_{obs} = \Delta C^\circ_{P,obs}\left[(T - T_H) - T\ln\left(\frac{T}{T_S}\right)\right] \quad (2)$$

$$\Delta H^\circ_{obs} = \Delta C^\circ_{P,obs}(T - T_H) \quad (3)$$

$$\Delta S^\circ_{obs} = \Delta C^\circ_{P,obs}\ln\frac{T}{T_H} \quad (4)$$

values of $\Delta C^\circ_{P,obs} = 1.9 \pm 0.1$ kcal/mol·K, $\Delta G^\circ_{obs}(22\ ^\circ C) = 10 \pm 2$ kcal/mol, $\Delta S^\circ_{obs}(22\ ^\circ C) = -18 \pm 4$ cal/mol·deg, and $\Delta H^\circ_{obs}(22\ ^\circ C) = 4.5 \pm 1$ kcal/mol were calculated. These values should be interpreted with caution since the nonlinear least-squares (MathCAD) fit of the data in Table II to eq 1 is very dependent upon the T_H and T_S values, which are difficult to determine within the limited precision of the measured dissociation constants. The fit of the data is shown in Figure 4A, and the thermodynamic parameters are plotted vs temperature in Figure 4B.

The decrease in K_D at higher temperature could also be due to thermal denaturation of the headpiece. Thus, the T_m of $\gtrsim 40\ ^\circ C$ (Hinz et al., 1981; Schnarr & Maurizot, 1982) for the headpiece is close enough to the temperature ($T > 25\ ^\circ C$) at which K_D increases with temperature. That both native repressor and headpiece both show a similar nonlinear dependence of the dissociation constant with temperature rules out the trivial explanation that the effect for the whole repressor is possibly attributable to the dissociation of individual subunits.

Salt Effect on lac Repressor Headpiece Binding to Operator DNA. The binding affinities of the wild-type *lac* repressor headpiece for the wild-type symmetric operator O1 were measured over 0.15–1.4 M salt concentrations (0.15 M NaCl plus additional KCl) using the alkaline phosphatase assay (Figure 5). The slope and correlation coefficient of the log K_D vs log (salt concentration) plot were 2.59 and 0.96, respectively. At lower salt concentrations (<0.35 M), the K_D is essentially independent of salt concentration with a limiting K_D of $\sim 10^{-7}$ M. Included in Figure 5 are the data from previous studies in the literature at ≤0.2 M salt concentrations.

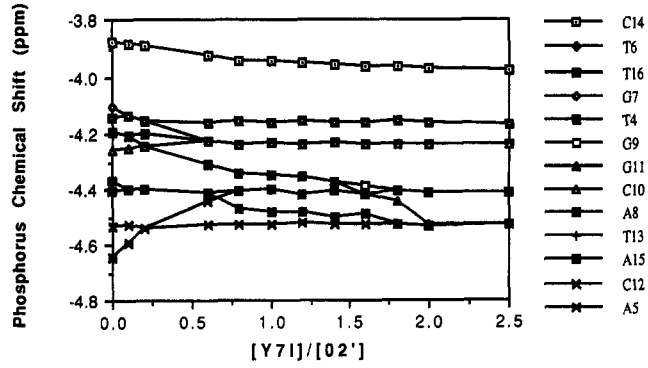

FIGURE 6: ^{31}P chemical study of the 14-bp oligonucleotide duplex, d(TATGAGCGCTCATA)$_2$ (mutant operator O2′), as a function of the relative ratio of [Y7I mutant *lac* repressor headpiece]/[14-bp O2′ operator]. Individual phosphates are identified in the figure.

Experimental results with *lac* repressor-operator binding performed by filter binding assays in low-salt conditions show a linear relationship when log [K_D] vs log [MX] is plotted between 0.05 and 0.2 M (Winter & von Hippel, 1981).

^{31}P NMR of Mutant Operator Repressor Complexes. The 14-bp symmetrical operator fragments are about two-thirds the length of the wild-type operator that has been identified by methylation protection (Ogata & Gilbert, 1979). However, the 14-mers are believed to contain most of the important recognition sites (Sadler et al., 1983; Simons et al., 1984) for the *lac* repressor protein. Mutagenesis studies (Betz et al., 1986; Lehming et al., 1987) have indicated that repressor protein still binds, although less strongly, to operators containing either a single-site G to A transition at position 5 (O2′), an A to T transversion at position 8 (O3′), or a G to C transversion at position 5 (O4′); note that, in keeping with the operator numbering convention in Figure 1, nucleoside and phosphate numbering in the 14-mers starts at residue 4 on the 5′-strand.

Our laboratory has previously assigned (Fu et al., 1988) the ^{31}P NMR spectrum of the 14-bp *lac* operator segment d(TGTGAGCGCTCACA)$_2$ (O1′), as well as three mutants, d(TATGAGCGCTCATA)$_2$ (O2′), d(TGTGTGCGCACACA)$_2$ (O3′), and d(TCTGAGCGCTCAGA)$_2$ (O4′), using either regiospecific ^{17}O-labeling of the phosphates (Schroeder et al., 1989) or 2D ^{31}P–^1H pure absorption phase constant time (PAC) heteronuclear correlation NMR spectroscopy.

The ^{31}P spectral changes upon binding the N-terminal 56-residue wild-type and Y7I mutant headpiece to all of the 14-mer operators (Karslake et al., 1990, 1992; Wisniowski et al., 1992) demonstrate that the phosphate resonances of the free and bound operator remain in fast chemical exchange during the entire course of the titration, because only one set of peaks is observed at all DNA/protein ratios [cf. Figure 13 in Wisniowski et al. (1992)]. At low salt concentrations, the ^{31}P chemical shift titration curves for all of the complexes level off at a ratio of two headpiece molecules (HP) per one operator duplex (O) [cf. Karslake et al. (1990) and Wisniowski et al. (1992)]. For example, a plot of the ^{31}P chemical shift vs [Y7I mutant HP]/[O2′ mutant operator] ratios for this complex is shown in Figure 6. Therefore, one headpiece is bound at each of the putative d(TGTGA) wild-type recognition sequences that are located on either side of the operator's 2-fold axis of symmetry.

A bar graph summary for the perturbations in the ^{31}P chemical shifts of the various operators upon formation of the 2:1 headpiece complexes for both wild-type and mutant repressors is shown in Figure 7. A comparison of the sequence dependence of ^{31}P chemical shifts of the wild-type or several

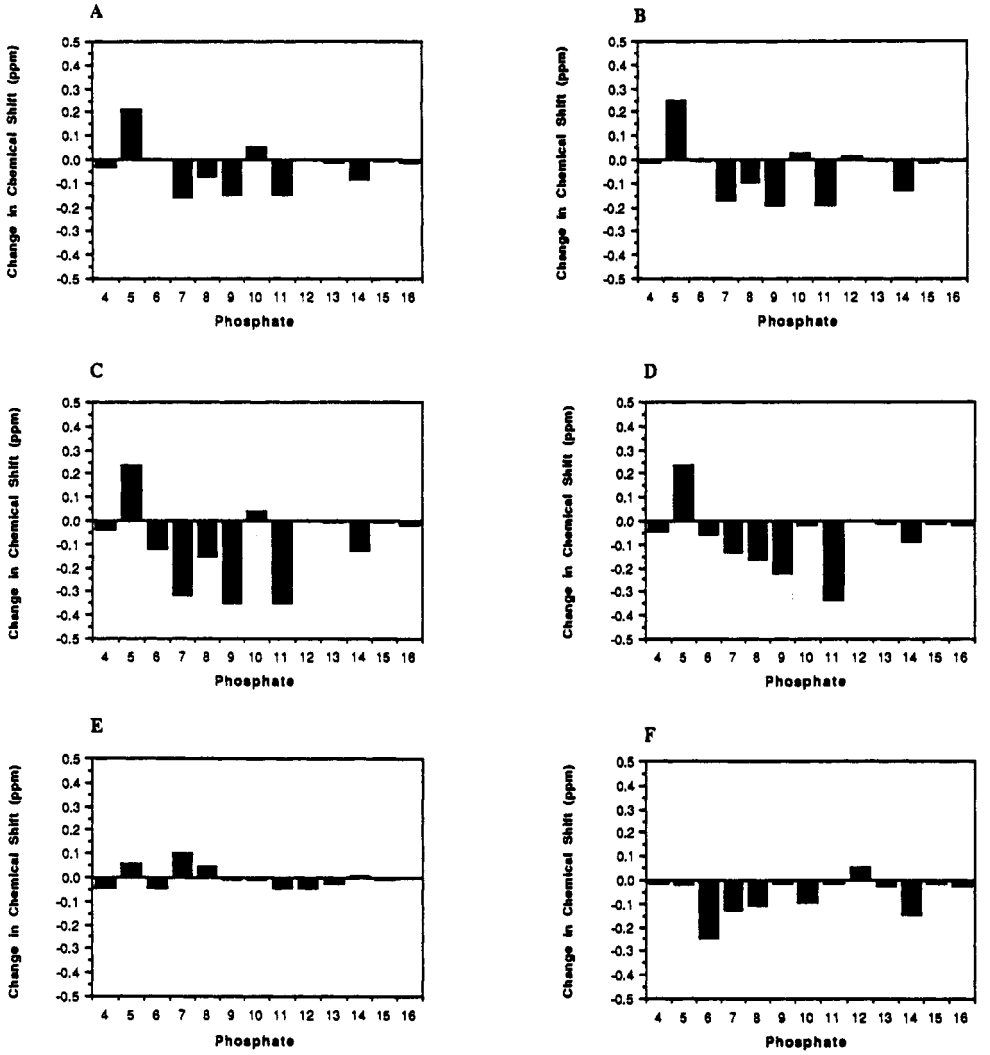

FIGURE 7: Summary bar plots of the perturbation of the ^{31}P chemical shift of individual phosphates of the 14-mer operators upon binding 2 equiv of the 56-residue *lac* repressor headpiece: (A) symmetric "wild-type" operator O1′ and wild-type headpiece; (B) wild-type operator O1′ and Y7I mutant headpiece; (C) O2′ mutant operator and wild-type headpiece; (D) O2′ mutant operator and Y7I mutant headpiece; (E) O3′ mutant operator; and (F) O4′ mutant operator and Y7I mutant headpiece. A–C are reproduced from Karslake et al. (1992) for comparison.

of the mutant operators bound to the wild-type or mutant headpieces is shown in Figure 8.

Titration of O2′ Operator with Headpiece. The perturbations of the ^{31}P chemical shifts of the O2′ operator upon the addition of either the mutant or the wild-type headpieces are very similar (Figure 7C,D). As found for the O2′ operator·wild-type headpiece complex (Figure 7C), Y7I interacts specifically with O2′ (Figure 7D), with the major ^{31}P changes occurring at phosphates 5, 7–9, and 11. Minor perturbations are also observed at phosphates 4, 6, and 14. The main difference between the two complexes of O2′ lies in the magnitude of the perturbation of phosphates 6, 7, 9, and 11. The changes in chemical shifts of phosphates 6 and 7 in the O2′ operator·wild-type headpiece complex are twice as large as those in the O2′ operator·Y7I headpiece complex. Moreover, whereas phosphates 9 and 11 are perturbed to the same degree by the wild-type headpiece, they are perturbed differently by the mutant headpiece. At the start of the titration, the signals of phosphates 9 and 11 are coincident. At a 1:1.6 O2′ operator/Y7I headpiece ratio, one of the phosphates merges with the signals of phosphates 13 and 15. Assuming that it is phosphate 11 that merges, we have shown in Figure 7D that phosphate 11 is perturbed by 0.35 ppm while phosphate 9 is perturbed by 0.22 ppm. The other case where it is phosphate 9 that merges is equally possible.

The pattern of ^{31}P chemical shift perturbations for the O2′ operator with the Y7I headpiece (Figure 7B) is also similar to that of the wild-type operator binding to the Y7I headpiece [Figure 4A in Karslake et al. (1992)]. In their unbound states, the chemical shifts of the phosphates of the wild-type and O2′ operators are quite comparable with the exception of phosphates 14 and 15. Presumably, O1′ and O2′ operators interact with the Y7I headpiece in a very similar fashion.

Titration of O3′ Operator with Headpiece. The titration of the O3′ operator with the Y7I headpiece gives small (0.05–0.1 ppm) but specific perturbations of phosphates 4–8, 11, and 12 (Figure 7E). This pattern of chemical shift perturbations is also observed when O3′ is titrated with the wild-type headpiece, with the exception of phosphates 7 and 8 which show somewhat smaller perturbations [Figure 3D in Karslake et al. (1992)].

In the titration of O3′ operator with either the wild-type or Y7I mutant headpiece, broadening of the phosphorus peaks is observed. This broadening suggests that the protein may be binding to multiple sites on the operator fragment. The mutation in O3′ changes Tp4Gp5Tp6Gp7Ap8Gp9 into Tp4Gp5Tp6Gp7*Tp8*Gp9. Since the headpiece appears to bind primarily to the TGTG site, the headpiece could interact with both the Tp4Gp5Tp6Gp7 and Tp6Gp7Tp8Gp9 sites. Binding to the latter site would physically preclude the binding of a

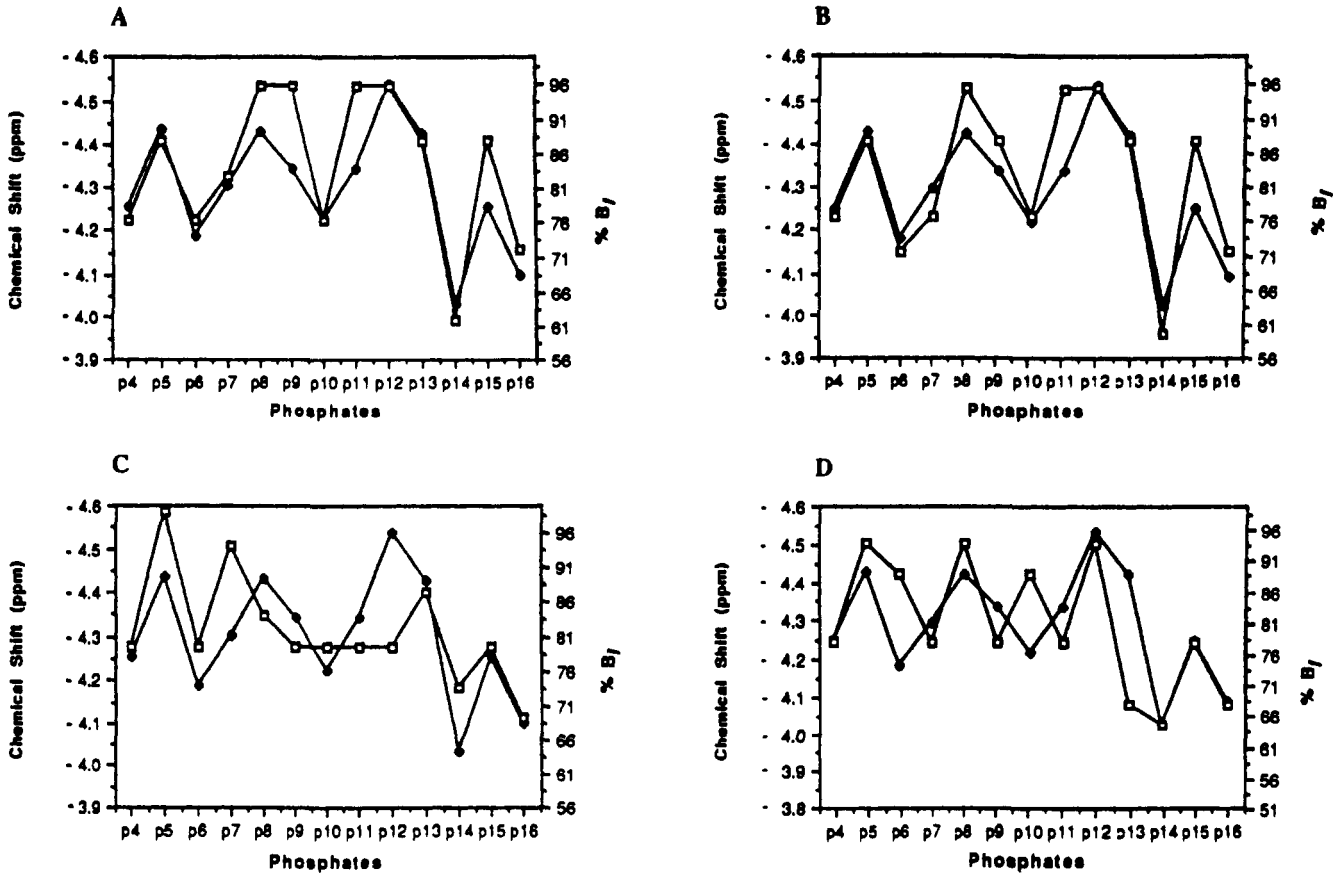

FIGURE 8: Plot of ^{31}P chemical shifts (□) vs sequence for (A) mutant O2′ operator·wild-type headpiece, (B) O2′ operator·Y7I mutant headpiece, (C) mutant O3′ operator·wild-type headpiece, and (D) mutant O4′ operator Y7I headpiece. The ^{31}P chemical shifts (◆) vs sequence for the wild-type O1 operator·wild-type headpiece are shown for comparison. The percentage of the B_I phosphate backbone conformation calculated from the ^{31}P chemical shifts as previously described (Roongta et al., 1990) is also shown. A and C are reproduced from Karslake et al. (1992) for comparison.

second headpiece at the other Tp4Gp5Tp6Gp7 site, resulting in a 1:1 complex.

Titration of O4′ Operator with Headpiece. While only very small perturbations within the reproducibility of the spectral changes and experimental error (<0.02 ppm) were observed for operator O4′ upon addition of the wild-type headpiece (Karslake et al., 1992), when O4′ operator is titrated with the Y7I headpiece, specific perturbations at phosphates 6–8, 10, and 14 (Figure 7F) are observed.

DISCUSSION

Although the results of a number of genetic studies suggest possible regions of contact between the *lac* repressor and operator (Goeddel et al., 1978; Khoury et al., 1991b; Lehming et al., 1987, 1988; Miller & Reznikoff, 1978; Sartorius et al., 1989, 1991; Takeda et al., 1983), the exact nature of the binding interaction on a molecular level is not known. However, 2D proton NMR is beginning to provide these details (de Vlieg et al., 1989; Zuiderweg et al., 1985). The repressor protein recognizes and binds strongly to the operator DNA, a unique 21-bp partially palindromic sequence of the *Escherichia coli* chromosome. The *lac* repressor protein binds to the operator section of DNA about 10 million times more strongly than to the rest of the *E. coli* chromosome (Berg et al., 1982). The 22-bp symmetrical operator O1 differs from the natural operator sequence by two A-T to G-C base-pair conversions on the right half of the operator, by deletion of a single G-C base pair at its center, and by addition of one flanking base pair at either end. These sequence alterations result in an 8–10-fold increase in repressor binding affinity over the natural operator sequence (Sadler et al., 1983). The shortened 14-bp completely palindromic operator segment d(TGTGAGCGCTCACA)$_2$ (O1′) is believed to contain most of the important contact sites (Sadler et al., 1983; Simons et al., 1984).

Binding of Repressors and Headpieces to Operators. The $K_{D(app)}$ values measured by a gel retardation assay for the *lac* wild-type and Y7I mutant repressors are 9.5×10^{-12} and 30.6×10^{-12} M, respectively (Wisniowski et al., 1992). This is comparable to other measured binding constants for the wild-type repressor and is consistent with previous reports (Kleina & Miller, 1990; Miller et al., 1979) that the tyrosine 7 to leucine mutant is a weak-binding repressor. The Y7I mutant repressor protein binds the 322-bp DNA fragment containing the wild-type nonpalindromic operator with only 3-fold poorer binding constant, and yet 2D NMR studies show that the mutation significantly disrupts the overall structure and stability of the recognition helix (Karslake et al., 1991; Wisniowski et al., 1992). In the mutant, loss of the Y7–Y17 aromatic side chain interaction, proposed to exist in the wild-type 56-residue headpiece, presumably selectively destabilizes the first two helices. The presence of nearly all of the tertiary structure cross-peaks (Karslake, 1991; Wisniowski et al., 1992) in the mutant, however, indicated that the overall folding was not dramatically altered.

It has not been possible to use gel retardation or filter binding assays to measure the binding of the N-terminal 56-residue *lac* repressor headpiece to the operator. However, the alkaline phosphatase assay has allowed us to measure dissociation constants in the 10^{-6}–10^{-8} M range. Dissociation constants,

K_D, in 0.35 M salt ranged from 5.4×10^{-8} M for wild-type repressor·wild-type symmetrical operator binding to $\gtrsim 1 \times 10^{-6}$ M for wild-type repressor·nonspecific DNA binding. Within experimental error, O2 and O4 bind nearly as tightly as the wild-type headpiece ($(6.4\text{-}7.0) \times 10^{-8}$ M), while O3 binds over 5-fold poorer (2.6×10^{-7} M).

Operators O1, O2, and O4 bind ca. 10-fold poorer to the Y7I mutant headpiece, with K_D's varying from 4.5×10^{-7} (O1 operator) to 6.7×10^{-7} M (for O4) (Table I). Thus, in contrast to the 3-fold change in the dissociation constant of the mutant holorepressor, the Y7I mutant headpiece bound various 22-bp symmetrical operator fragments ca. 10-fold poorer than the wild-type headpiece. Presumably the difference can be attributed to the higher specificity of the wild-type repressor (and headpiece) for the left-hand (TGTGA) side of the nonpalindromic wild-type operator. If we neglect entropy effects and assume that each subunit of the *lac* repressor binds to the left-hand and right-hand sides of the symmetrical operator independently, then a headpiece should bind with an association constant approximately equal to the square root of that of the intact repressor (Ogata & Gilbert, 1979; $K_{dimer} \approx K^2_{monomer}$). Even if we correct for the 10-fold tighter binding of the repressor to the completely palindromic operator, the monomer headpiece appears to have an intrinsically higher affinity (ca. 5.4×10^{-8}) than is simply predicted from the measured binding constant of the wild-type repressor for the 322-bp fragment containing the nonpalindromic operator ($(0.1 \times 10^{-12})^{1/2}$). This could suggest that some of the intrinsic binding energy of the whole repressor is utilized to distort the structure of the DNA upon binding. Indeed, distortion such as bending of a DNA fragment by binding the 434 repressor has been observed (Mondragon & Harrison, 1991).

Ogata and Gilbert (1979) reported dissociation constants of $(3.5\text{–}5) \times 10^{-6}$ and $(2.5\text{–}6.5) \times 10^{-5}$ M for the 55-bp-long restriction fragment containing the wild-type unsymmetrical *lac* operator complexed to the N-terminal 59 and 51 amino acid fragments of the wild-type *lac* repressor, respectively. The dissociation constants were determined by methylation protection experiments at guanines 5 and 17 (where the protection was strongest). The conditions for the Ogata and Gilbert dissociation constant measurements were slightly different from those of our own assay. Repeating these buffer conditions but using our alkaline phosphatase assay for the wild-type 56 residue headpiece and our 22-bp palindromic "super"operator, we measure a K_D of 3.5×10^{-7} M. The ca. 10-fold tighter binding compared to the Ogata and Gilbert value is at least partially explained by our use of the superoperator. Employing a similar methylation experiment, Khoury et al. (1991) also noted strongest protection at guanine 5 in the *lac* UV promoter·operator region in the presence of the 56 and 64 amino acid *lac* repressor fragments. The level of protection was comparable to that observed by Ogata and Gilbert. Although Khoury et al. (1991) did not report the dissociation constants for these headpieces, values in the micromolar range were mentioned.

Comparing previously measured K_D's with ours (5.4×10^{-8} M for the wild-type 56-mer headpiece·wild-type symmetrical operator), we see a 10–100-fold tighter binding in our complex. The difference is thus attributed to the use of the symmetric operator as well as different buffer conditions. Our results confirm the utility of the alkaline phosphatase assay for measuring dissociation constants in the difficult to quantify range of 10^{-6}–10^{-9} M.

Salt Effects on lac Repressor and Repressor Headpiece Binding. DNA is a highly charged polyanion, with two negative charges per 3.4 Å along the DNA sugar phosphate backbone. This high negative charge density causes the accumulation of cations and the exclusion of anions along the DNA polymer. In the polyanion condensation theory (Record et al., 1991), DNA behaves like a weak electrolyte and an ion concentration gradient effectively neutralizes the DNA charge. In the polyanion condensation theory, the univalent salt (MX) dependence on K_{obs} for protein·DNA interactions is given as

$$\frac{\partial \ln K_{obs}}{\partial \ln [MX]} = a$$

where a represents the cations released upon the protein binding to the DNA. Experimental results with *lac* repressor·operator binding performed by filter binding assays in low-salt conditions show a linear relationship when log $[K_D]$ vs log [MX] is plotted between 0.05 and 0.2 M (Winter & von Hippel, 1981; data reproduced in Figure 5). Typically, values of $-a$ of 5–20 are observed (Goeddel et al., 1977; Record et al., 1991; Winter & von Hippel, 1981). For specific *lac* operators, Record and co-workers have obtained values of 5 ± 1 for interaction with the *lac* repressor, although one mutant operator showed a value for $-a$ of 10. For interaction of nonspecific DNA with the *lac* repressor, $-a = 11 \pm 1$ (deHaseth et al., 1977a,b; Revzin & von Hippel, 1977).

The binding affinities of the wild-type *lac* repressor headpiece to the wild-type symmetric operator O1 were also measured from 0.15 to 1.40 M salt using the alkaline phosphatase assay (Figure 5). The slope of the log K_D vs log (salt) plot ($-a = 2.59$) is in good agreement with the work of Goeddel et al. (1977), who reported a value of 2 for whole wild-type repressor binding to 21- and 26-bp *lac* operators. It has been suggested that the smaller number of cations released could be attributed to the absence of flanking sequences which could be involved in the charge·charge interactions present in the protein·DNA interaction. Also, it has been reported that phosphates located near the ends of DNA fragments bind counterions less effectively than do those in the middle of a long DNA strand, and thus show less counterion displacement on repressor binding (Record & Lohman, 1978).

^{31}P NMR. The perturbations in ^{31}P chemical shifts in forming the operator-headpiece (O·HP) complex can arise from several sources. Electrostatics and local shielding effects by the bound protein certainly can play a role (Gorenstein, 1992). However, our laboratory has demonstrated that the phosphate ester conformation plays a dominant role in the ^{31}P chemical shift differences in small DNA fragments (Roongta et al., 1990). We have shown that for free DNA duplexes (even including some base-pair mismatches, bulge DNA, and drug DNA·complexes) there exists a very good correlation between measured ^{31}P chemical shifts and $J_{H3'-P}$ coupling constants (Roongta et al., 1990). From the latter, we can calculate the C4′–C3′–O–P torsional angle ($\epsilon = -\theta - 120°$) and infer information about C3′–O3′–P–O5′ (ζ) (Gorenstein, 1992; Roongta et al., 1990; Schroeder et al., 1989). These studies have shown that most phosphates are instantaneously in a B_I conformational state ($\epsilon = 180°$ or t and $\zeta = -60°$ or g^-), while others are in a B_{II} conformational state ($\epsilon = g^-$, $\zeta = t$). A rapid, picosecond time scale or faster "jump"-interconverts these two low-energy phosphate ester conformations, and most phosphate esters populate both the B_I and B_{II} states. Sequence-specific variations in the conformation of the DNA sugar phosphate backbone thus can possibly

explain the sequence-specific recognition of DNA, as mediated through direct contacts and electrostatic complementarity between the phosphates and the protein (Karslake et al., 1992).

The ^{31}P chemical shift of a phosphodiester in a pure B_I or B_{II} conformational state has been estimated to be −4.6 and −3.0 ppm, respectively (Roongta et al., 1990). The dispersion in the ^{31}P chemical shifts of oligonucleotides is thus likely attributable to sequence-specific variations in the ratios of populations of the B_I and B_{II} states for each phosphate in the sequence. We have assumed that the ^{31}P chemical shifts in the HP·O complexes also vary as a direct result of changes in the relative populations of the B_I and B_{II} states. We can then calculate the fractional populations by assuming a simple two-state model derived from the analysis of coupling constants and ^{31}P chemical shifts in the free duplex data. This population axis is also shown in Figure 8.

^{31}P NMR of Wild-Type and Mutant Operator Complexes. In all of the complexes the range of ^{31}P chemical shifts in the free operators narrows upon binding either wild-type or mutant headpiece as the most upfield signal shifts downfield and the furthest downfield signal moves upfield. This presumably reflects a change to a more uniform conformation (more B_I-like) for all of the phosphates in all of the operator headpiece complexes. Consistent with this interpretation note that in the O1′, O2′, and O3′ operators phosphate 5 is shifted *downfield* upon binding to either wild-type or Y7I headpiece (in O1′ and O2′ complexes it is the only phosphate that is shifted significantly downfield). In each of the O1′–O3′ operators, phosphate 5 is also the most upfield signal. In contrast, in the O4′ operator, phosphate 5 is not the most upfield and it shifts slightly upfield or not at all in the complexes.

The interaction of O2′ mutant operator with the wild-type headpiece as monitored by ^{31}P NMR spectroscopy is also very similar to that of wild-type headpiece and wild-type O1′ operator. The phosphate sites for this interaction are the same as those sites observed for the wild-type·wild-type operator/repressor interaction. However, one difference is that the magnitude of the change in chemical shifts is greater for the O2′ interaction than for any others that have been studied so far. This is really quite remarkable because the mutant O2′ operator binds more weakly to both the wild-type and Y7I mutant headpieces. Thus the magnitude of the ^{31}P perturbations is *not* reflective of the tightness of the complex.

As previously observed for sequence-specific ^{31}P chemical shift effects in the free operators, the ^{31}P chemical shifts are largely perturbed only at the sites surrounding the mutation (Schroeder et al., 1989). Phosphates that are more than two positions removed generally have ^{31}P shifts identical to those of the wild-type sequence. Thus in the free mutant O2′ operator, the ^{31}P chemical shifts of only phosphates p14 and p15 (and to a lesser extent, p6 and p7) differ significantly from those of operator O1′, even though the G5 → A5 (and C16 → T16) mutations might be expected to perturb nearest-neighbor phosphates p4–p7 and p14–p16. This indicates that the conformational states of all of the phosphates in O2′ are very similar to their counterparts in O1′, with the exception of p6, p7, p15, and p16. However, as shown by the bar plots of Figure 7A–F, the ^{31}P chemical shifts of p15 and p16 are little changed in any of the complexes, suggesting that the protein makes little contact with these phosphates.

In the free mutant O3′ operator, ^{31}P chemical shifts mainly differ at phosphates p7 and p12 (and to a lesser extent at p8–p10) from the free O1′ operator, although the mutation sites are adjacent to p7/p8 and p12/p13. Although p12 and p14 have been shown to play minor roles in backbone recognition, p7 remains a major site of interaction. Alteration of the conformation of this phosphate in the free operator appears to have a significant effect on the magnitude of the perturbations in ^{31}P chemical shifts on forming the headpiece·O3′ complex [Figure 7E; see also Figure 3D in Karslake et al. (1992)]. Indeed, the pattern of ^{31}P chemical shifts vs sequence of the wild-type headpiece complex with O3′ compares very poorly with shifts of the wild-type headpiece·O1′ complex (Figure 8C). Thus while the basic variations in chemical shifts vs sequence in the wild-type headpiece complex with either O2′ or O1′ are quite comparable (Figure 8A), the pattern of chemical shifts vs sequence for the O3′·wild-type headpiece complex matches poorly (Figure 8C).

Table III: Number of B_I Phosphates in Native and Mutant *lac* Repressor·Operator Complexes

	repressor headpiece[a]	
operator	wild type	Y7I
O1′	1 (−1)	2 (0)
O2′	4 (2)	3 (1)
O3′	2[b] (0[b])	1[b] (−1[b])
O4′	2 (0)	3 (1)

[a] Numbers in parentheses represent the increase in the number of B_I phosphates in the complex (positive number) relative to the free duplex. [b] Data for 1:1 complexes and results are thus not comparable to the other operators that bind as a 2:1 complex.

The G5 to C5 (and C16 to G16) transversion in the free O4′ mutant significantly perturbs the ^{31}P chemical shifts (and conformation) of the phosphates p13 and p14 (and to a lesser extent p4–p6) relative to the free O1′ operator. There is no specific perturbation in the chemical shifts of the O4′ operator upon formation of the wild type (changes in chemical shifts <0.02 ppm). However, moderate perturbation in the chemical shifts of the O4′ operator is observed upon binding to the mutant headpiece (Figure 7F). This is the only operator in which the perturbation is greater for the mutant than for the wild-type repressor headpiece. Although this mutation is in the middle of the putative TGTG recognition site, the lack of ^{31}P chemical shift changes upon addition of the headpiece does not reflect weak binding to this mutant operator. As shown in Table I, O4′ binds nearly as well to the wild-type headpiece as O1′. The wild-type and mutant operators also bind to Y7I with similar affinities.

As we had previously noted (Karslake et al., 1992), in the *lac* mutant operator·headpiece complexes, several of the ^{31}P chemical shifts of phosphates are significantly perturbed from those of the wild-type HP·O1′ (or Y7I·O1′) complex (Figure 8). If we assume that the ^{31}P chemical shift changes reflect conformational state changes (and of course other variables can contribute to these chemical shift changes; Gorenstein, 1992), phoshates such as p8, p9, and p11 in the O2′ complex are apparently contrained to a B_I conformation (Figure 8B; p15 is also shifted to a more B_I-type state). The criterion used for a constrained B_I-type state is the existence of a fractional population of >91% B_I. As shown in Table III, only the wild-type O1′ operator·wild-type headpiece has a single phosphate constrained to a B_I state (because the O3′ operator binds as a 1:1 complex and because of the complexity of intermediate chemical exchange broadening the spectra of the complex, we ignore this complex in this analysis). All of the other complexes listed in Table III have 2–4 phosphates constrained to a more B_I-like state. This is a reflection that almost all of the ^{31}P signals are shifted upfield (more B_I-like), as shown in the bar plots of Figure 7. This is also consistent

with the change in the number of B_I phosphates for each operator upon formation of the complex (numbers in parentheses, Table 3). Interestingly, the wild-type operator shows one less constrained B_I phosphate upon formation of the complex.

As shown by Mossing and Record (1985), the free energy difference between the in vitro binding of the *lac* repressor protein and either the wild-type operator or the position 5 G to A transition mutant in the left side of the operator is ca. 4 kcal/mol. A similar loss of in vivo repression by repressor binding to a palindromic O2′-type mutant at complementary positions 5 (G to A) and 16 (C to T) has also been observed (Lehming et al., 1987). In vivo symmetric O2–O4 bind >70-fold poorer to the whole *lac* repressor than the wild-type O1 operator (Lehming et al., 1987). Although the experimental errors in the dissociation constants shown in Table I make comparison between the operators and headpieces more difficult, a similar trend is observed. Thus O2′ appears to bind more weakly to the wild-type headpiece than O1′ (indeed among all of the operators, the headpiece binds most tightly to the wild-type O1′ operator). However, why the magnitudes of the differences are so small in the complexes is perplexing.

Record and co-workers have shown that a significant driving force at lower temperatures for forming the *lac* repressor·operator complexes in entropic (Ha et al., 1989), which we now find is indeed also true for the *lac* repressor headpiece (Figure 4B). Because the temperature dependence of the dissociation constants is nonlinear for both intact repressor and headpiece (Figure 4A), the entropy change and the enthalpy change are strongly temperature dependent. This is reflected in a large heat capacity change, $\Delta C^{\circ}_{p,obs}$. A number of explanations have been offered for processes involving large $\Delta C^{\circ}_{p,obs}$ values (Spolar et al., 1989; Sturtevant, 1977). Record and co-workers suggest that a major factor in protein·DNA association is attributable to a hydrophobic effect, representing a change in the water-accessible surface area of nonpolar groups.

We have proposed that an additional, hitherto unrecognized, mechanism for discrimination between operators is based upon the degree to which the repressor protein restricts phosphate ester conformational freedom in the complex (Karslake et al., 1992). We suggested that specific, strongly bound complexes *retain the inherent phosphate ester conformational flexibility of the operator itself*, whereas more weakly bound, but still specific, operator·protein complexes restrict the phosphate ester conformational freedom in the complex relative to the free DNA. This torsional effect would correspond to changes in internal vibrational modes that could be contributing to the large heat capacity changes.

This proposal, based upon the loss of conformational freedom in the complex contributing to the specificity of interaction, can also explain the 10-fold reduction in the binding of Y7I mutant to wild-type operator. The first two helices of the Y7I mutant headpiece are partially disordered (Karslake et al., 1991), which is not the case for the wild-type headpiece. In order for the second, recognition helix to bind within the major groove of the operator, this disorder or dynamic flexibility must be removed. Conformational restriction of the peptide backbone will be entropically unfavorable, presumably reflected in the weaker association of the mutant headpiece. (In the wild-type headpiece, the recognition helix is already conformationally restricted and no entropy must be lost in order for it to fold and bind to the operator.) Similar ideas have been offered to explain aspects of protein·protein recognition (Lumry, 1974).

Thus, our data suggest that optimal protein·DNA recognition will require a balance between the dynamics of both the protein and the DNA. Too rigid of a protein will lock up the phosphates, reducing affinity. If the protein is too flexible, then a certain amount of entropy will be lost as the secondary structure is ordered to allow operator binding and recognition. This requirement for retention of DNA and protein backbone torsional freedom in strongly bound complexes (which is entropically favorable) provides a new mechanism for protein discrimination of different operator binding sites. Thus upon binding, various rotational degrees of freedom must be lost if the repressor is to bind to only one of the phosphate conformations. The dissociation constant will reflect this internal entropic disadvantage, which may be as large as 8 eu (2.4 kcal/mol at 25 °C) per lost degree of torsional freedom (Page & Jencks, 1971). For freezing two rotational degrees of freedom, this entropic factor may represent a binding difference of up to 10^3–10^4. In duplex DNA, of course, the phosphodiester bonds of the different phosphates are already partially conformationally constrained, and the entropic penalty will be much less. At a minimum for a two-state system, the entropic factor will be RT ln 2 or 0.4 kcal/mol at room temperature. X-ray crystal structures, solution NMR structures, and molecular dynamics simulations demonstrate that the most conformationally mobile portion of the DNA is the phosphate ester (Gorenstein, 1992). Binding of the repressor in such a way as to restrict the intrinsic conformational freedom of 20 or more of the operator phosphates could, in principle, contribute to a sizable entropic disadvantage.

This analysis suggests another explanation for the entropy differences in the binding of various wild-type and specific mutant operators for repressor as well an entirely new appreciation for the difficulty in which a protein must recognize the strucutral features *and dynamics* of operator DNA. Thus in the strongly bound, specific complexes, the protein must not only provide a binding surface that matches the sequence-specific variation in the phosphate conformation of the operator but also allow for retention of the phosphate conformational freedom in the complex. The position of the phosphate will move in changing from a B_I to a B_{II} state, and indeed the position of the phosphate shows the greatest RMS variation in molecular dynamics calculations (Pearlman & Kollman, 1991). This will place enormous constraints on the protein structure in the complex if the phosphate ester conformational freedom is not to be greatly reduced in the complex. This can only be possible if the protein·DNA interface is flexible enough and if there is a "coupling" of the motion of the amino acid residues in the binding site with the phosphate ester motion. In contrast, in a specific mutant operator that does not bind as strongly to the repressor, the subtle structural and dynamical requirements for providing the necessary coupling or flexibility are presumably lost so as to preclude free torsion of at least some of the phosphate esters in the protein·DNA complex. Recall that in the wild-type O1′·wild-type HP complex the ^{31}P data suggest that only one of the phosphates appears to be restricted to a B_I state. In contrast, in all of the mutant complexes (operators and/or headpiece), 2–4 phosphates are "frozen" in a B_I conformational state (Table III).

Therefore, we have suggested that a significant requirement for an "evolutionarily perfected" repressor to strongly bind a specific operator is to allow sufficient flexibility in the complex such that the phosphate can still jump between the B_I and B_{II} states. It should not be so flexible that in its free state the recognition helix is disordered. In the crystal structure of

several protein·DNA complexes, water appears in the interface between the protein and DNA (Anderson et al., 1987; Otwinowski et al., 1988), which is generally not found in inhibitor·enzyme complexes. Note that water molecules and sodium ions cannot be distinguished in the crystal structures. It is quite reasonable that the intervening water or counterion serves as a mobile partner, which allows for the retention of much of the phosphodiester backbone flexibility in the protein·DNA complex. In mutant operators and proteins, presumably the structural and dynamical coupling between the protein and DNA is disrupted such that the DNA and/or the protein backbone conformational freedom in the complex is greatly restricted through either steric or electrostatic interactions.

ACKNOWLEDGMENT

We thank Dr. Paul Wisniowski for early contributions to this work, Dr. Joan Betz for the plasmid, and Pei Ling Wang for help in the protein purification.

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