Seligy, V. L., & Poon, N. H. (1978) *Nucleic Acids Res. 5*, 2233–2252.
Simpson, R. T. (1979) *J. Biol. Chem. 254*, 10123–10127.
Simpson, R. T., & Bergman, L. W. (1980) *J. Biol. Chem. 255*, 10702–10709.
Subirana, J. A. (1973) *J. Mol. Biol. 74*, 363–386.
Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell Biol. 83*, 403–427.
Touchette, N. A., & Cole, R. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2642–2646.
Touchette, N. A., Anton, E., & Cole, R. D. (1986) *J. Biol. Chem. 261*, 2185–2188.
Vinograd, J., Lebowitz, J., & Watson, R. (1968) *J. Mol. Biol. 33*, 173–177.
Weischet, W., Tatchell, K., van Holde, K. E., & Klump, H. (1978) *Nucleic Acids Res. 5*, 139–160.

# $^{31}$P NMR Spectra of Oligodeoxyribonucleotide Duplex *lac* Operator–Repressor Headpiece Complexes: Importance of Phosphate Ester Backbone Flexibility in Protein–DNA Recognition†

Christine Karslake, Maria Victoria Botuyan, and David G. Gorenstein*

*Department of Chemistry, Purdue University, West Lafayette, Indiana 47907*

*Received September 9, 1991; Revised Manuscript Received November 15, 1991*

ABSTRACT: The $^{31}$P NMR spectra of various 14-base-pair *lac* operators bound to both wild-type and mutant *lac* repressor headpiece proteins were analyzed to provide information on the backbone conformation in the complexes. The $^{31}$P NMR spectrum of a wild-type symmetrical operator, d(TGTGAGCGCTCACA)$_2$, bound to the N-terminal 56-residue headpiece fragment of a Y7I mutant repressor was nearly identical to the spectrum of the same operator bound to the wild-type repressor headpiece. In contrast, the $^{31}$P NMR spectrum of the mutant operator, d(TATGAGCGCTCATA)$_2$, wild-type headpiece complex was significantly perturbed relative to the wild-type repressor–operator complex. The $^{31}$P chemical shifts of the phosphates of a second mutant operator, d(TGTGTGCGCACACA)$_2$, showed small but specific changes upon complexation with either the wild-type or mutant headpiece. The $^{31}$P chemical shifts of the phosphates of a third mutant operator, d(TCTGAGCGCTCAGA)$_2$, showed no perturbations upon addition of the wild-type headpiece. The $^{31}$P NMR results provide further evidence for predominant recognition of the 5′-strand of the 5′-TGTGA/3′-ACACT binding site in a 2:1 protein to headpiece complex. It is proposed that specific, strong-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 strong complexes is entropically favorable and provides a new (and speculative) mechanism for protein discrimination of different operator binding sites. It demonstrates the potential importance of phosphate geometry and flexibility on protein recognition and binding.

How do proteins recognize DNA? Most attention on 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). Thus, although the *lac* repressor protein does not recognize an alternating AT sequence as part of the *lac* operator DNA sequence, the repressor protein binds to poly[d(AT)] 1000 times more strongly than to random DNA (Saenger, 1984). Repressor protein is quite likely recognizing the alternating deoxyribose–phosphate backbone geometry of the two strands (Klug et al., 1979), rather than the chemical identity of the AT base pairs.

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 appears to be 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

† This work was 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).

* Author to whom correspondence should be addressed.

0006-2960/92/0431-1849$03.00/0 

the *lac* repressor headpiece as well as headpiece–operator fragment complexes by 2D NMR methods (Wüthrich, 1986). The $^1$H spectrum of a Tyr 7 to Ile mutant *lac* repressor headpiece has also been assigned (Karslake et al., 1991; Wisniowski et al., 1991). NOESY distance-restrained molecular dynamics studies of repressor headpiece bound to *lac* operator DNA fragments have begun to provide details confirming the sequence-specific interactions of a recognition $\alpha$-helix binding within the major groove of the operator DNA.

Remarkably, in every high-resolution X-ray crystal structure of a repressor–operator complex, the majority of the contacts are to the phosphates [Jordan & Pabo, 1988; Otwinowski et al., 1988; Wolberger et al., 1988; see also Kissinger et al. (1990) and Schultz et al. (1991)]. Indeed, the crystal structure of the *trp* repressor demonstrated that every one of the direct protein contacts were mediated through interactions with the phosphate backbone [Otwinowski et al. (1988), however, some controversy exists as to the correctness of this complex; Marmorstein et al. (1991) and Staacke et al. (1990)]. Ionic interactions involving the phosphate backbone have been implicated as being important factors in the recognition of the *lac* operator as well (deHaseth et al., 1977; Karslake et al., 1990). It is not known whether any of these ionic interactions provide a specific recognition mechanism for these repressors.

Phosphorus NMR can potentially provide information on protein–DNA phosphate backbone interactions because $^{31}$P chemical shifts provide direct information on the conformation of the phosphate ester (Karslake et al., 1990; Roongta et al., 1990; Schroeder et al., 1989). Thus phosphates in a gauche$^-$ ($g^-$; dihedral angle, –60°) conformation about one of the P–O ester bonds have a $^{31}$P chemical shift ca. 1.6 ppm upfield of the phosphates in a trans (t; dihedral angle 180°) conformation (Gorenstein, 1991; Roongta et al., 1990). Local helical parameters such as helix twist and base-pair roll appear to affect the $^{31}$P chemical shift by stretching or contracting the sugar–phosphate backbone, which in turn alters the time-averaged conformation of the phosphate ester. Therefore, sequence-specific information about the DNA is potentially contained within the geometry of the phosphates and may be obtained from the $^{31}$P NMR spectrum. As described in this paper, by analysis of the $^{31}$P spectral changes of small operator fragments upon binding wild-type and mutant *lac* repressor headpieces, we suggest that the conformation and, in particular, the flexibility of the phosphates may be an important component of protein–DNA recognition.

## Experimental Procedures

*Synthesis.* The self-complementary 14-base-pair *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), complementary sites of mutation in the palindromic operators are in boldface, were synthesized by a manual modification of the solid-phase phosphite triester method (using 10 $\mu$mol of the starting nucleoside derivatized support at a 28 mmol/g loading level for each separate synthesis) as previously described (Schroeder et al., 1987). The sequence of the operators and numbering scheme for the phosphates is shown in Figure 1. The resulting tritylated product was cleaved from the support and purified by C-18 reverse-phase HPLC with an acetonitrile/triethylammonium acetate buffer gradient as previously described (Gorenstein et al., 1988). The amounts of purified oligonucleotides obtained through one or more separate syntheses were 600–900 OD units.

*Y7I Mutant lac Repressor Headpiece.* Plasmids pHIQ3 and pHIQ6 and *Escherichia coli* strain MC1061 were gifts from Dr. Joan Betz. This overproducing strain with the pHIQ3 plasmid provided ca. 200 mg of wild-type *lac* repressor protein per 100 L of culture. The Tyr 7 to Ile (Y7I) mutant *lac* repressor was constructed by oligonucleotide-directed site-specific mutagensis (Karslake et al., 1991; Wisniowski et al., 1991) on the plasmid pHIQ6.

O1

$T_{p4}G_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}C_{p16}A$

O2

$T_{p4}A_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}\mathbf{T}_{p16}A$

O3

$T_{p4}G_{p5}T_{p6}G_{p7}T_{p8}G_{p9}C_{p10}G_{p11}C_{p12}\mathbf{A}_{p13}C_{p14}A_{p15}C_{p16}A$

O4

$T_{p4}C_{p5}T_{p6}G_{p7}A_{p8}G_{p9}C_{p10}G_{p11}C_{p12}T_{p13}C_{p14}A_{p15}G_{p16}A$

FIGURE 1: Symmetric 14-mer wild-type operator O1 and mutant operators O2–O4.

*Wild-Type and Y7I Mutant lac Repressor Headpiece Purification.* The N-terminal 56-residue headpieces were prepared according to Arndt et al. (1981) as previously described for the wild-type protein (Karslake et al., 1990) with the additional quenching of the $\alpha$-chymotrypsin (Worthington) digest with a 4 molar equivalent excess of turkey trypsin inhibitor (Sigma). Approximately 120 mg of mutant repressor was obtained from 300 g of wet cell paste compared to 220 mg of wild-type repressor prepared from 300 g of wet cell paste containing the plasmid pHIQ3. (The pHIQ3 plasmid contains two copies of the repressor protein encoding gene.) The wild-type and mutant headpieces were characterized by amino acid analysis, bio-ion Cf desorption mass spectroscopy and 2D NMR (Karslake et al., 1991; Wisniowski et al., 1991).

*Preparation of $^{31}$P NMR Samples.* $^{31}$P NMR samples were prepared by dissolving 4–6 mg of the lyophilized DNA in 0.4 mL of 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 headpiece and DNA concentrations were measured by UV spectroscopy. Amounts of the 14-mers were determined spectrophotometrically using the relationship of 20 absorbance units (optical density, OD, units) per 1.0 mg of DNA at 260 nm.

*$^{31}$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 to the signal-to-noise ratio in the $^{31}$P NMR spectrum. The number of acquisitions for each spectrum were typically between 2000 and 3000. The $^{31}$P resonances were referenced to an external sample of tri-

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

## RESULTS

*Operator–Headpiece $^{31}P$ NMR Spectra.* Our laboratory has previously assigned (Fu et al., 1988) the $^{31}P$ NMR spectrum of the 14-base-pair *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 $^{31}P$–$^{1}H$ pure absorption phase constant time (PAC) heteronuclear correlation NMR spectroscopy (Figure 1).

These symmetrical base sequences are about two-thirds the length of the 21-base-pair wild-type sequence, and 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 in keeping with operator numbering convention, nucleoside and phosphate numbering in the 14-mers starts at residue 4 on the 5′-strand.

It is possible to duplicate the basic *lac* operator–*lac* repressor protein interaction by using the smaller *lac* repressor headpiece N-terminal domain fragment (Buck et al., 1978; Wade-Jardetzky et al., 1979). The $^{31}P$ spectral changes upon binding the N-terminal 56-residue headpiece to all of the 14-mer operators [cf. Figure 2 and Karslake et al. (1990)] 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. At low ionic strength, 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); spectra not shown]. Therefore, one headpiece is bound at each of the putative dTGTGA recognition sequences that are located on either side of the operator's 2-fold axis of symmetry. At higher ionic strength, we had previously observed that only one wild-type headpiece is bound per O1 operator as measured by the $^{31}P$ shift titration curve (Karlake et al., 1990).

Because of peak overlap during various additions of headpiece to the operator, it is necessary to clarify how the phosphate resonances were assigned during the titration. We assumed that perturbations in the $^{31}P$ chemical shift were a continuous function throughout the titration. Thus peaks that did not shift with added protein remained unshifted once the peak had merged with another peak. In addition, the slope of the perturbation was assumed to stay constant during merging of the peaks.

In the wild-type O1 operator binding to the wild-type headpiece (Karslake et al., 1990), the $^{31}P$ signals of phosphates 5, 7, and 8 are well resolved at the beginning of the titration and show significant perturbation upon addition of headpiece (the G5 phosphate shifts 0.20 ppm downfield while the G7 phosphate shifts 0.16 ppm upfield during the titration). The A8 phosphate shifts 0.13 ppm upfield during the titration. Unfortunately the $^{31}P$ signals of phosphates 6, 9, and 11 are coincident in the absence of repressor, and in the 2:1 complex two of the three shift 0.15 ppm upfield during the titration. By integration of the spectra, it was determined that two of

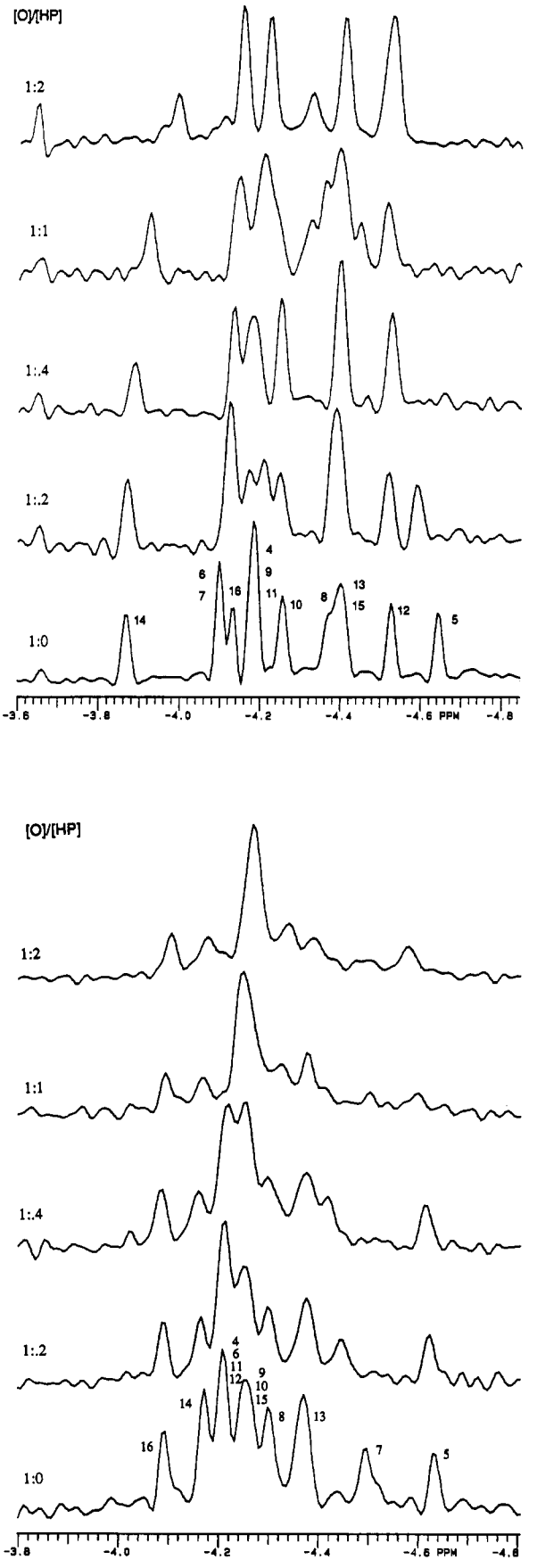


FIGURE 2: $^{31}P$ NMR spectra of the 14 bp oligonucleotide duplex (A, top) d(TATGAGCGCTCATA)$_2$ (mutant operator O2) and (B, bottom) d(TGTGTGCGCACACA)$_2$ (O3) as a function of the relative ratio of [14 bp operator]/[wild-type *lac* repressor headpiece] at 0.067 M ionic strength. The [O]/[HP] ratios during the titration are shown. See Figure 1 for numbering of the phosphates shown.

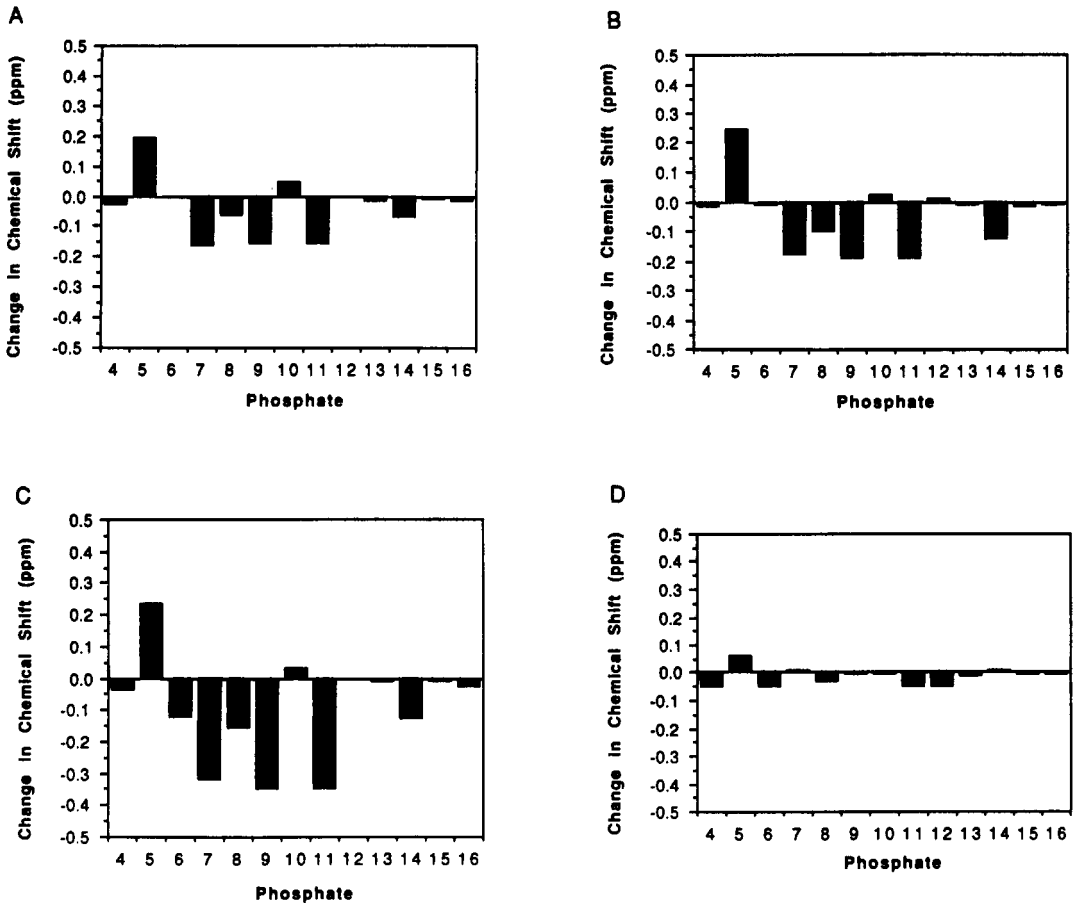


FIGURE 3: Summary bar plots of the perturbation of the $^{31}P$ chemical shift of individual phosphates of the 14-mer operators upon binding 2 equiv of the 56-residue *lac* repressor headpiece: (A) symmetric "wild-type" operator O1 and wild-type headpiece; (B) wild-type operator O1 and Y7I mutant headpiece; (C) O2 mutant operator and wild-type headpiece, and (D) O3 mutant operator and wild-type headpiece.

these three phosphates were perturbed upon addition of the wild-type headpiece. Because it was impossible to determine which two of the three peaks were perturbed, it was assumed earlier that resonances 6 and 9 were perturbed because they were adjacent to other phosphates which were perturbed during the titration. However, the $^{31}P$ signals of phosphates 9 and 11 in mutant O2 are resolved from resonance 6, and the titration data of O2 with wild-type headpiece would suggest another interpretation. Both the resonances arising from phosphates 9 and 11 are perturbed during this titration, and the resonance of phosphate 6 is unaffected. Assuming that the wild-type headpiece interacts in a similar manner with both the wild-type operator O1 and the mutant O2 operator, we propose that the phosphates of 9 and 11 and not 6 interact with the wild-type headpiece. The $^{31}P$ signals in O1 of the remaining phosphates 4, 10, and 12–16 show either no or small perturbations (<0.1 ppm) upon titration with wild-type headpiece. Assuming that the magnitude of the $^{31}P$ shift reflects the degree of interaction of the phosphate with the headpiece, we can conclude that phosphates 5–8 (and possibly 9) represent a major binding site for the headpiece. A revised bar graph figure for the perturbations in the $^{31}P$ chemical shifts of the wild-type operator upon formation of the 2:1 wild-type headpiece complex is shown in Figure 3A. A summary of these $^{31}P$ chemical shift perturbations for several other complexes are shown in the bar graphs of Figure 3B–D.

The pattern of changes in the $^{31}P$ chemical shifts of the wild-type symmetrical operator upon binding a Tyr 7 to Ile (Y7I) mutant headpiece are strikingly similar to the changes observed for the wild-type headpiece–wild-type operator complex (Figures 3A, B and 4A). The Y7I mutant repressor protein binds a 322 bp DNA fragment containing the wild-type nonpalindromic operator with only a 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 [residues 17–25; see Karslake et al. (1991) and Wisniowski et al. (1991)]. 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 helix. The presence of nearly all of the tertiary structure cross-peaks (Karslake, 1991; Wisniowski et al., 1991) in the mutant indicates that the overall folding has not been dramatically altered. As found for the wild-type operator–wild-type headpiece complex, the interaction of the Y7I mutant headpiece with "wild-type" operator is highly specific with the only major $^{31}P$ changes occurring at phosphates 5, 7, 8, 9, and 11. Smaller perturbations are also observed for phosphates 6 and 14.

The pattern of $^{31}P$ chemical shift perturbations shown by the bar plot for the O2 mutant operator with wild-type headpiece (Figure 3C) is also similar to that for wild-type operator O1 with either headpiece (Figures 3A, B). Because the chemical shifts of the phosphates in the free O2 operator are quite comparable to those of O1 (with the exception of the p14 and p15 phosphates), the sequence-specific variation in the $^{31}P$ chemical shifts of the O·HP complexes are also in general similar (with the exceptions of p8, p9, p11, and p15; Figure 4B).

The titration of O3 operator was shown to cause severe broadening of the phosphorus peaks at higher protein to DNA ratios. While the specific perturbations of any of the phosphorus resonances were quite small, the final 2:1 complex

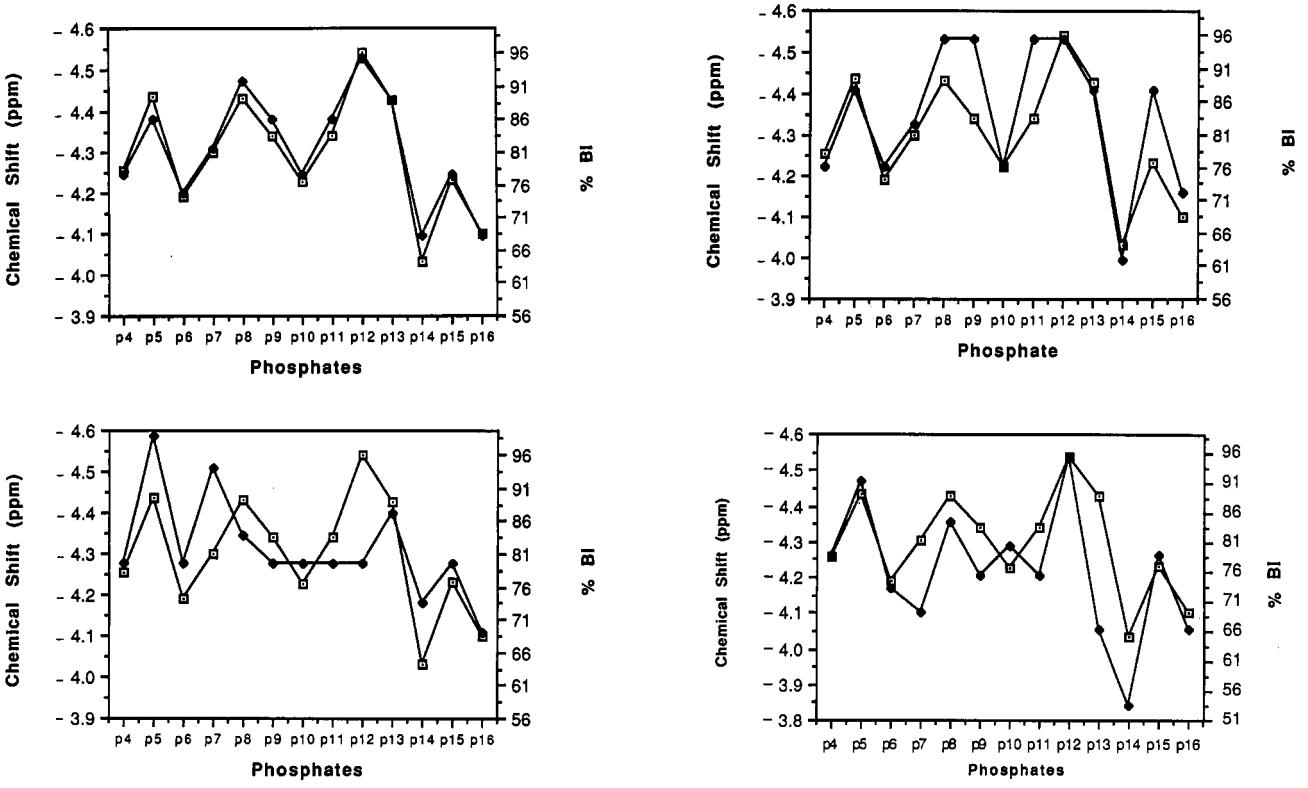


FIGURE 4: Plot of $^{31}P$ chemical shifts (◆) vs sequence for (A, top left) wild-type O1 operator–Y7I mutant headpiece (B, top right) mutant O2 operator–wild-type headpiece (C, bottom left) mutant O3 operator–wild-type headpiece, and (D, bottom right) mutant O4 operator–wild-type headpiece. The $^{31}P$ chemical shifts (⊡) vs sequence for the wild-type O1 operator–wild-type headpiece is 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.

pattern for both wild-type HP and the Y7I mutant is in general similar to those of O1 and O2 (cf. Figure 3D). This broadening suggests that the protein may be binding to multiple sites on the operator fragment. All of the peaks broadened to such a large extent that it is possible that some of the peaks in the complex may be misassigned. In several cases the peaks were broadened, causing a merging of several previously well-resolved resonances. The mutation in O3 changes Tp4Gp5Tp6Gp7Ap8Gp9 into Tp4Gp5Tp6Gp7*Tp8*Gp9. Since 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 second headpiece at the other Tp4Gp5Tp6Gp7 site, resulting in a 1:1 complex.

Only nonspecific, very small perturbations within the reproducibility of the spectral changes and experimental error (<0.02 ppm) were observed for operator O4 upon addition of either wild-type or mutant headpiece.

## Discussion

Although the results of a number of genetic studies suggest possible regions of contacts between the *lac* repressor and operator (Groeddel et al., 1978; Khoury et al., 1991; 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. The repressor protein recognizes and binds strongly to the operator DNA, a unique 21-base-pair sequence of the *E. 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 14-base-pair completely palindromic *lac* "pseudooperator" segment d(TGTGAGCGCTCACA)$_2$ (referred to as the "wild-type" operator, O1, in this paper) is believed to contain most of the important contact sites (Sadler et al., 1983; Simons et al., 1984). The pseudooperator differs from the natural operator sequence by two A–T to G–C base-pair conversions on the right half of the operator and by deletion of a single G–C base pair at its center. These sequence alterations result in an 8- to 10-fold increase in repressor binding affinity over the natural operator sequence (Sadler et al., 1983).

X-ray crystallographic structural refinements, extensive mutagenesis experiments (Betz, 1986, 1987; Ebright, 1986; Eisenbeis et al., 1985; Koudelka et al., 1987; Lehming et al., 1987, 1988; Wharton et al., 1984; Wharton & Ptashne, 1985), as well as model-building on a number of operator DNA complexes, including *lac* (de Vlieg et al., 1989), suggest that certain side-chain residues recognize the individual bases of nucleic acids, while others "recognize" the backbone phosphates, possibly through sequence-specific variations in the DNA conformation. We have suggested (Karslake et al., 1990) that $^{31}P$ NMR spectra can be a useful spectroscopic probe of the backbone DNA interactions.

The $^{31}P$ spectral changes upon binding the 56-residue wild-type or Y7I mutant headpiece to the wild-type or mutant operators demonstrate that all of the phosphate resonances remain in fast exchange during the entire course of the titration. At low ionic strength, the $^{31}P$ titration of the various headpiece and operator curves level off at a ratio of two headpiece molecules (HP) per one operator duplex (O) [cf. Karslake et al. (1990)]. Therefore, one headpiece is bound at each of the putative dTGTGA recognition sequences that are located on either side of the operator's 2-fold axis of symmetry.

The $^{31}P$ chemical shift perturbations induced by binding the Y7I headpiece to wild-type operator are almost identical to those observed for binding the wild-type headpiece to the wild-type operator. It appears that the Y7I mutant and the

wild-type headpiece both interact to the same degree with the wild-type operator, largely at phosphates p5–p9. The Y7I mutant repressor has been shown by band-shift assay to bind the natural operator in a 322 bp *Pvu*II fragment from pUC19 approximately 3-fold less strongly than the wild-type repressor. The solution structure of this 56-residue mutant headpiece is currently being determined in our laboratory (Karslake et al., 1991; Wisniowski et al., 1991). Preliminary data suggest that this mutant repressor has very similar structure compared to the wild-type headpiece as determined by the medium- and long-distance NOEs seen in the two headpieces. However, the first and second helices of the Y7I mutant have been shown to be partially disrupted. We have also begun NMR structural studies on the Y7I headpiece complexed with the 14-base-pair operator (Wisniowski et al., 1991). The preliminary analysis confirms NOEs from isoleucine-7 side-chain protons of the mutant headpiece to the O1 operator, similar to the interactions observed for Y7 in the wild-type headpiece.

*Origin of $^{31}$P Chemical Shift Differences and Perturbations.* The perturbations in $^{31}$P chemical shifts in forming the O–HP complex can arise from several sources. Electrostatics and local shielding effects by the bound protein certainly can play a role (Gorenstein, 1991). 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 a P–H3′ Karplus coupling constant/torsional angle relationship (Lankhorst et al., 1984), we can calculate the C4′–C3′–O–P torsional angle. As shown by Dickerson (Dickerson, 1983; Dickerson & Drew, 1981), there is a strong correlation ($R$ = –0.92) between torsional angles C4′–C3′–O3′–P ($\epsilon$) and C3′–O3′–P–O5′ ($\zeta$) in the crystal structures of various duplexes. A similar correlation appears to exist in solution as well (Gorenstein, 1991; Roongta et al., 1990; Schroeder et al., 1989). Thus, both torsional angles $\epsilon$ and $\zeta$ can often be calculated from the measured P–H3′ coupling constant and directly related to the measured $^{31}$P chemical shift. These studies have shown that most phosphates are instantaneously either in "$B_I$" conformational state (Figure 5) ($\epsilon$ = 180° or t and $\zeta$ = –60° or g$^-$) while others are in a "$B_{II}$" conformational state ($\epsilon$ = g$^-$ and $\zeta$ = t). A rapid, picosecond time scale or faster "jump" interconverts these two low-energy phosphate ester conformations and most phosphate esters populate both $B_I$ and $B_{II}$ states. It is important to point out that these coupled changes in the torsional angles $\epsilon$ and $\zeta$ related to the $B_I$ to $B_{II}$ conformational transition rotate the orientation of the phosphoryl oxygens relative to the helix axis. The plane formed by the phosphorus atom and the phosphoryl oxygens of the phosphate in the $B_I$ conformation is nearly perpendicular to the plane formed by the phosphorus atom and the phosphoryl oxygens of the phosphate in the $B_{II}$ conformation.

The populations of the $B_I$ and $B_{II}$ conformational states appear to be controlled by sequence-specific variation in the local helical structure which can alter the length of the sugar–phosphate backbone (Schroeder et al., 1989). Thus, if the helix unwinds, the length of the sugar–phosphate backbone between a base step must contract. In turn, this alters the conformation of the phosphate. 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. 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.

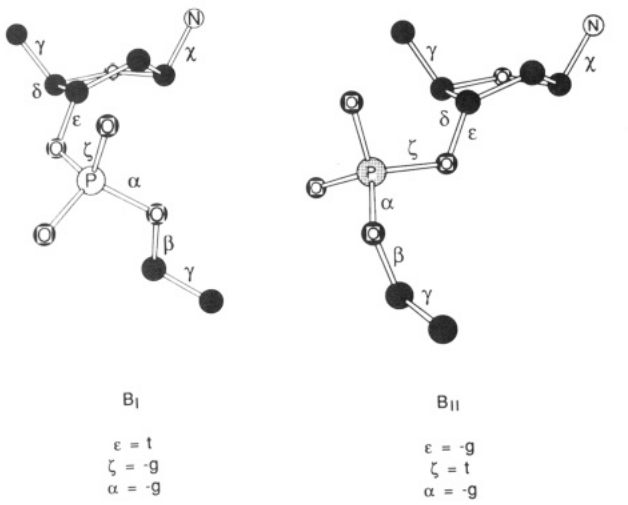


FIGURE 5: Views of sugar–phosphate backbone in $B_I$ (left) and $B_{II}$ (right) conformational states. $\alpha$, $\beta$, $\epsilon$, $\zeta$: g$^-$, t, t, g$^-$ ($B_I$); g$^-$, t, g$^-$, t ($B_{II}$). The $B_I$ conformation represents the low-energy phosphate state normally observed in the crystal structures for many of the duplex phosphates, and the $B_{II}$ conformation represents the other major sugar–phosphate state for B-DNA. Torsional angles: gauche$^-$ (g$^-$ or –60°); trans (180°). Crystal structures of duplex oligonucleotides show that these angles are only approximate, and indeed the $\zeta$ angle is generally closer to –90° for what is defined as "g$^-$".

We now assume 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 4. Interestingly, ab initio molecular orbital calculations have estimated the energy difference between a gauche ($B_I$-like) and a trans ($B_{II}$-like) conformation about the phosphodiester bond to be approximately 1 kcal/mol (Gorenstein, 1987). A Boltzmann distribution would then predict that the $B_I$ conformation in a random coil DNA is approximately 81% populated at room temperature. Remarkably, as shown by the $^{31}$P NMR data and using the two-state model analysis, we calculate an average population of the phosphates in the HP–O1 complex as ca. 84% $B_I$. Thus, in the wild-type complex the average phosphate ester conformation is quite comparable to that of an unconstrained phosphate diester.

*Mutant Operator Complexes.* The interaction of O2 mutant operator with 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 of interaction for this interaction are the same as those sites observed for the wild-type–wild-type operator–repressor interaction. However, one important difference is that the magnitude of the change in chemical shifts is about twice as large for the O2 operator as for the wild-type O1 operator. This might seem a bit paradoxical with the more tightly bound wild-type operator showing much smaller perturbations upon binding than the mutant operator O2.

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 identical $^{31}$P shifts as 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 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 state 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 3A–E, 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 the backbone recognition, p7 remains a major site of interaction. Changing 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 3D). Indeed the pattern of $^{31}P$ chemical shifts vs sequence of the wild-type headpiece complex with O3 very poorly compares to shifts of the wild-type headpiece–O1 complex (Figure 4C). Thus while the basic variation of chemical shifts vs sequence in the wild-type headpiece complex with either O2 or O1 are quite comparable (Figure 4B), the pattern of chemical shifts vs sequence for the O3–wild-type headpiece complex match poorly (Figure 4C).

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, p5, and 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 or Y7I mutant complex (changes in chemical shift <0.02 ppm). This mutation is in the middle of the putative TGTG recognition site, and the lack of $^{31}P$ chemical shift changes upon addition of headpiece is thus likely reflecting the weak binding to this mutant operator. The basic mismatch between the phosphate conformational states (defined by the $^{31}P$ chemical shifts) of the O4–HP and O1–HP complexes is conveyed by Figure 4D.

de Haseth et al. (1977) have previously determined that the phosphates play an important role in the recognition of *lac* operator by its repressor. They showed that approximately 12 phosphates were involved in this interaction. Also, deVlieg et al. (1989) have modeled the interaction of *lac* repressor headpiece and a 14-base-pair nonsymmetric operator with the 51 amino acid wild-type headpiece using NOESY distance restraints obtained by Boelens et al. (1987). On the basis of these NOESY distance-restrained molecular dynamics calculations in a box of water, they noted that many of the protein–DNA contacts were to phosphate groups. Specifically, there were direct contacts to phosphates 5, 12, and 14 and a few other phosphates which are not contained in our pseudooperator. We are observing phosphate perturbation at phosphate 5 and a smaller perturbation at 14 but none at phosphate 12.

The relative binding affinities of a number of site-specific mutants of the *lac* repressor (Betz, 1986, 1987; Ebright, 1986; Lehming et al., 1987, 1988; Sartorius et al., 1989, 1991) to various wild-type and mutant operators provide support for the model proposed by Kaptein and van Gunsteren and coworkers (Boelens et al., 1987; de Vlieg et al., 1989). The data of Lehming et al. (1987, 1988) confirm that the major recognition site of the operator is the 5′-TGTGA sequence starting at the first guanosine. Müller-Hill and co-workers' mutagenesis studies (Sartorius et al., 1989) on the repressor protein have implicated p4Gp5, p5Tp6, and p6Gp7 as sites of contact. Our results also indicate that phosphate p5 through p9 are involved in the recognition of the operator DNA.

*Hypothesis: Importance of Phosphate Ester Backbone Flexibility in Protein–DNA Recognition.* In the *lac* O2 (and to a lesser extent, O3) mutant operator–headpiece complexes, several of the $^{31}P$ chemical shifts of phosphates are significantly perturbed from those of the HP–O1 (or Y7I–O1) complex. Assuming that the $^{31}P$ chemical shift changes reflect conformational state changes [and of course other variables can contribute to these chemical shift changes (Gorenstein, 1991)], phosphates such as p8, p9, and p11 in the O2 complex are apparently constrained to a $B_I$ conformation (Figure 4B; p15 is also shifted to a more $B_I$-type state).

Using the equilibrium constants for the fractional populations of the $B_I/B_{II}$ states for each phosphate, the change in free energy differences between the HP–O2 complex and the HP–O1 complex (comparing only the differing phosphates p8, p9, p11, and p15) is approximately 3 kcal/mol. 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). The entropy of binding of wild-type operator with wild-type repressor is ca. 100 eu at room temperature (Mossing & Record, 1985) whereas the entropy of binding the O2-type mutant operator is only 90 eu. The difference in entropy of binding between the O2-type and wild-type operators is ca. 10 eu (3 kcal/mol), and thus the weaker binding of an O2-type mutant appears to be largely an entropic factor.

Record and co-workers have postulated that a significant amount of the increase in entropy in forming the complexes is attributable to release of bound cations and water molecules. We propose 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. We speculate 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 requirement for *retention* of 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 were 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. However, 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, 1991). 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 both 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 structural 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 is 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 O2–HP complex the $^{31}P$ data suggests that several of the phosphates appear to be restricted to a $B_I$ state.

Note, these arguments for the importance of retention of phosphate backbone conformational freedom in the complex are independent of the time scale of the "motion". Entropy, as a state function, is unaffected whether within a given complex the backbone undergoes rapid (perhaps even picosecond motion, as in the free DNA) jumps between the $B_I$ and $B_{II}$ states or whether the complex must dissociate (over minutes in the case of the wild-type *lac* repressor) and then reassociate to give a new complex with a differently constrained conformational state for one or more phosphates. The reassociated complex can of course arise from a different repressor molecule. It is the ensemble of molecules that is important, although the meaning of this in a cell with a single wild-type operator site is an intriguing question. All that is required is that in a strongly bound complex, both the $B_I$ or $B_{II}$ state at each phosphate can be bound, thus retaining the original conformational freedom of the phosphates in the free operator. In less strongly bound but specific complexes (e.g., O2), most of the phosphates still retain their freedom, while a few phosphates are constrained to one, for example $B_I$, conformational state, presumably due to unfavorable steric or Coulombic interactions at these backbone sites. In a nonspecific complex (such as O4), enthalpic contributions to binding are too weak to perturb the equilibrium population of conformers for any of the phosphates. Only in a moderately strongly bound, specific mutant complex will this constraint of backbone freedom in one or more phosphates contribute to a reduced binding constant. This view of protein–DNA flexibility still requires that the protein–DNA interaction surface permit several alternatively bound phosphate ester conformations.

Indeed the concept of the importance of restriction of conformational freedom in protein complexes is of course not new and is invoked frequently to explain enzymatic catalysis. However, in an enzyme, intrinsic binding energy is used to constrain the substrate to a conformation that in some way resembles the transition state (Page & Jencks, 1971) and to lower the activation energy for the reaction. Of course there is an entropic disadvantage to the constraint of a substrate in the enzyme–substrate complex into a single reactive conformation, but these are offset by the intrinsic binding energy of the substrate for the enzyme. A poorer, nonspecific substrate is not as tightly constrained by the enzyme to the reactive conformation because there is not sufficient intrinsic binding energy to cancel the unfavorable entropic restriction in conformational freedom in the bound substrate. Our proposed mechanism for protein–DNA recognition is exactly the opposite of the expected effect in an enzyme complex. An operator that is bound strongly specifically retains this conformational freedom, whereas an operator that is less strongly bound, but still specifically bound, does not. A DNA backbone that is frozen in the complex (to a $B_I$ or perhaps in some cases to a $B_{II}$ conformation) of course would entail significant entropic penalties for constraining the otherwise conformationally mobile phosphodiester backbone.

Therefore, we speculate 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. 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, presumably the structural and dynamical coupling between the protein and DNA is disrupted such that the backbone conformational freedom in the complex is greatly restricted through either steric or electrostatic interactions. This constrains one or more of the phosphodiester bonds, as shown by the O2 and O3 mutant operator–headpiece complexes. This is reflected in significant entropic penalties, resulting in poorer binding to the repressor. Finally, in a nonspecific, weakly bound operator such as O4, the very poor complementarity and poor interaction between the protein and both base-pair and phosphate ester backbone will also result in no restrictions on the backbone flexibility.

## Conclusions

Do DNA-binding proteins recognize these sequence-specific backbone structural and dynamical features of DNA? It is not unrealistic to assume that at least a portion of protein–DNA recognition derives from recognition of the sequence-specific variation in the position, geometry, *and* dynamics of the DNA phosphates (an indirect readout mechanism). The conformational populations of the phosphate esters directly reflects the local helical geometry and base sequence. Further, DNA is not a uniform cylinder of equivalently placed negative charges but rather one in which the phosphate charges can significantly migrate across the surface of the cylinder in response to changes in sequence-specific local helical parameters. Certainly electrostatic and hydrogen-bonding interactions can be a major factor in stabilizing these complexes (Jordan & Pabo, 1988; Otwinowski et al., 1988; Wolberger et al., 1988; Kissinger et al., 1990; Schultz et al., 1991). Finally, DNA is a dynamic molecule, especially in the phosphodiester backbone. Perhaps understanding the "second genetic" code will require that we more fully appreciate the

role of conformational flexibility in defining protein–DNA interactions.

ACKNOWLEDGMENTS

We thank Dr. Steve Schroeder, Dr. Joan Betz, and Pei Ling Wang.

REFERENCES

Adler, K., Beyreuther, K., Fanning, E., Geisler, N., Gronenborn, B., Klemm, A., Müller-Hill, B., & Pfahl, M. (1972) *Nature 72*, 322–327.

Anderson, J. E., Plashne, M., & Harrison, S. C. (1987) *Nature 326*, 846–852.

Arndt, K. T., Boschelli, F., Lu, P., & Miller, J. H. (1981) *Biochemistry 20*, 6109–6118.

Berg, O. G., Winter, R. B., & Von Hippel, P. H. (1982) *Trends Biochem. Sci. 7*, 52.

Betz, J. L. (1986) *Gene 42*, 283–292.

Betz, J. L. (1987) *J. Mol. Biol. 195*, 495–504.

Betz, J. L., Sasmor, H., Buck, F., Insley, M., & Caruthers, M. (1986) *Gene 50*, 123–132.

Boelens, R., Scheek, R. M., vanBoom, J. H., & Kaptein, R. (1987) *J. Mol. Biol. 193*, 213–234.

Buck, F., Rüterjans, H., & Beyreuther, K. (1978) *FEBS Lett. 78*, 335–338.

Buck, F., Rüterjans, H., Kaptein, R., & Beyreuther, K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5145–5148.

Buck, F., Hahn, K., Zemann, W., Rüterjans, H., Sadler, J. R., Breyreuther, K., Kaptein, R., & Scheek, R. (1983) *Eur. J. Biochem. 132*, 321–327.

deHaseth, P. L., Lohman, T. M., & Record, Jr., M. T. (1977) *Biochemistry 16*, 4783–4790.

de Vlieg, J., Berendsen, H. J. C., & van Gunsteren, W. F. (1989) *Proteins: Struct., Funct., Genet. 6*, 104–127.

Dickerson, R. E. (1983) *J. Mol. Biol. 166*, 419–441.

Dickerson, R. E., & Drew, H. R. (1981) *J. Mol. Biol. 149*, 761–786.

Ebright, R. H. (1986) *Protein Structure Folding and Design* (Oxender, D., Ed.) Alan R. Liss, pp 207–219, New York.

Eisenbeis, S. J., Nasoff, M. S., Noble, S. A., Bracco, L. P., Dodds, D. R., & Caruthers, M. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1084–1088.

Fu, J. M., Schroeder, S. A., Jones, C. R., Santini, R., & Gorenstein, D. G. (1988) *J. Magn. Reson. 77*, 577–582.

Gorenstein, D. G. (1987) *Chem. Rev. 87*, 1047–1077.

Gorenstein, D. G. (1991) *DNA Structures, Methods in Enzymology* (Lilley, D. M., & Dahlberg, J. E., Eds.), Academic Press, Inc., Orlando, FL.

Gorenstein, D. G., Schroeder, S. A., Fu, J. M., Metz, J. T., Roongta, V. A., & Jones, C. R. (1988) *Biochemistry 27*, 7223–7237.

Groeddel, D. V., Yansura, D. G., & Caruthers, M. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3578–3582.

Hogan, M., Wemmer, D., Bray, R. P., Wade-Jardetzky, N., & Jardetzky, O. (1981) *FEBS Lett. 124*, 202–203.

Jordan, S. R., & Pabo, C. O. (1988) *Science 242*, 893–899.

Karslake, C. A. (1991) Ph.D. Thesis, Purdue University, West Lafayette, IN.

Karslake, C., Schroeder, S., Wang, P. L., & Gorenstein, D. G. (1990) *Biochemistry 29*, 6578–6584.

Karslake, C., Wisniowski, P., Spangler, B. D., Moulin, A.-C., Wang, P. L., & Gorenstein, D. G. (1991) *J. Am. Chem. Soc. 113*, 4003–4005.

Khoury, A. M., Nick, H. S., & Lu, P. (1991) *J. Mol. Biol. 220*, 1–12.

Kissinger, C. R., Liu, B. S., Martinblanco, E., Kornberg, T. B., & Pabo, C. O. (1990) *Cell 63*, 579–590.

Klug, A., Viswamitra, M. A., Kennard, O., Shakked, Z., & Steitz, T. A. (1979) *J. Mol. Biol. 131*, 669–680.

Koudelka, G. B., Harrison, S. C., & Ptashne, M. (1987) *Nature 326*, 886.

Lamerichs, R. M. J. N., Boelens, R., van der Marel, G. A., van Boom, J. H., Kaptein, R., Buck, F., Fera, B., & Rüterjans, H. (1989) *Biochemistry 28*, 2985–2991.

Landschulz, W. H., Johnson, P. F., & McKnight, S. L. (1988) *Science 240*, 1759–1764.

Lankhorst, P. P., Haasnoot, C. A. G., Erkelens, C., & Altona, C. (1984) *J. Biomol. Struct. Dyn. 1*, 1387–1405.

Lehming, N., Sartorius, J., Niemöller, M., Genenger, G., von Wilcken-Bergmann, B., & Müller-Hill, B. (1987) *EMBO J. 6*, 3145–3153.

Lehming, N., Sartorius, J., Oehler, S., von Wilcken-Bergmann, B., & Müller-Hill, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7947–7951.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 466–467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marmorstein, R. Q., Sprinzl, M., & Sigler, P. B. (1991) *Biochemistry 30*, 1141–1148.

Matthews, B. W. (1988) *Nature 335*, 294–295.

Miller, J. H., & Reznikoff, W. S. (1978) *The Operon*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mossing, M. C., & Record, M. T., Jr. (1985) *J. Mol. Biol. 186*, 295–305.

Nick, H., Arndt, K., Boschelli, F., Jarema, M.-A. C., Lillis, M., Sadler, J. R., Caruthers, M., & Lu, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 218–222.

Otwinowski, Z., Schevitz, R. W., Zhang, R. G., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., & Sigler, P. B. (1988) *Nature 335*, 321.

Page, M. I., & Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678–1683.

Pearlman, D. A., & Kollman, P. A. (1991) *J. Mol. Biol. 220*, 457–479.

Record, Jr., M. T., deHaseth, P. L., & Lohman, T. M. (1977) *Biochemistry 16*, 4791–4796.

Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67–83.

Roongta, V. A., Powers, R., Nikonowicz, E. P., Jones, C. R., & Gorenstein, D. G. (1990) *Biochemistry 29*, 5245–5258.

Sadler, J. R., Sasmore, H., & Betz, J. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6785–6789.

Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.

Sartorius, J., Lehming, N., Kister, B., von Wilcken-Bergmann, B., & Müller-Hill, B. (1989) *EMBO J. 8*, 1265–1270.

Sartorius, J., Lehming, N., Kisterswoike, B., von Wilcken-Bergmann, B., & Müller-Hill, B. (1991) *J. Mol. Biol. 218*, 313–321.

Scheek, R. M., Zuiderweg, E. R. P., Klappe, K. J. M., van-Boom, J. H., Kaptein, R., Rüterjans, H., & Beyreuther, K. (1983) *Biochemistry 22*, 228–235.

Schroeder, S. A., Fu, J. M., Jones, C. R., & Gorenstein, D. G. (1987) *Biochemistry 26*, 3812–3821.

Schroeder, S. A., Roongta, V., Fu, J. M., Jones, C. R., & Gorenstein, D. G. (1989) *Biochemistry 28*, 8292–8303.

Schultz, S. C., Shields, G. C., & Steitz, T. A. (1991) *Science 253*, 1001–1007.

Simons, A., Tils, D., von Wilcken-Bergmann, B., & Müller-Hill, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1624–1628.

Staacke, E., Walter, B., Kisters-Woike, B., Wilken-Bergmann, B., & Müller-Hill, B. (1990) *EMBO J. 9*, 1963–1967.

Takeda, Y., Ohlendorf, D. H., Anderson, W. F., & Matthews, B. W. (1983) *Science 221*, 1020-1026.
Wade-Jardetzky, N., Bray, R. P., Conover, W. W., Jardetzky, O., Geisler, N., & Weber, K. (1979) *J. Mol. Biol. 128*, 259-264.
Wemmer, D., & Kallenbach, N. R. (1983) *Biochemistry 22*, 1901.
Wharton, R. P., & Ptashne, M. (1985) *Nature 316*, 601-605.
Wharton, R. P., Brown, E. L., & Ptashne, M. (1984) *Cell 38*, 361-369.
Whitson, P. A., Olson, J. S., & Matthews, K. S. (1986) *Biochemistry 25*, 3852-3858.
Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6948-6960.
Wisniowski, P., Karslake, C., Piotto, M. E., Spangler, B., Moulin, A.-C., Nikonowicz, E. P., Kaluarachchi, K., & Gorenstein, D. G. (1991) *J. Biomol. Struct. Stereodyn.* (in press).
Wolberger, C., Dong, Y., Ptashne, M., & Harrison, S. C. (1988) *Nature 335*, 789-795.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Zuiderweg, E., Scheek, R., Boelens, R., van Gunsteren, R., & Kaptein, R. (1985) *Biochimie 67*, 707-715.

# cGMP Phosphodiesterase Dependent Light-Induced Scattering Changes in Suspensions of Retinal Disc Membranes

Nelly Bennett* and Armel Clerc

*Laboratoire de Biophysique Molêculaire et Cellulaire, Unitê de Recherche Associêe 520 du Centre National de la Recherche Scientifique, Centre d'Etudes Nuclêaires de Grenoble, BP 85X, 38041 Grenoble Cedex, France*

*Received May 31, 1991; Revised Manuscript Received September 25, 1991*

ABSTRACT: Light-induced GTP-dependent scattering changes are studied in suspensions of retinal disc membranes to which one or both of the purified proteins involved in the phototransduction mechanism (G-protein and cGMP phosphodiesterase) are reassociated; a scattering change which depends on the presence of both G-protein (G) and inhibited cGMP phosphodiesterase (PDE) and on an ATPase-dependent process, previously described in Bennett [(1986) *Eur. J. Biochem. 157*, 487-495] is compared to the signal observed in the absence of PDE or of ATP and to PDE activity. The same signal can also be induced either in the dark or in the light by addition of preactivated G in the presence of inhibited PDE. This PDE-dependent scattering change is composed of two components (fast and slow); the variation of the amplitude and kinetics of both components with PDE or G concentration is similar to the variation of the active PDE state with two activator $G_{GTP}$ molecules (G with GTP bound), calculated with dissociation constants previously reported for the interaction between $G_{GTP}$ and PDE [Bennett, N., & Clerc, A. (1989) *Biochemistry 28*, 7418-7424]. The two components are therefore proposed to be associated with processes which depend on the formation of the active PDE state with two activators.

Absorption of a photon by the photosensitive protein rhodopsin in retinal discs leads to the closing of sodium channels in the outer membrane of the rod. The channels are kept open in the dark by direct binding of cGMP, the concentration of which is rapidly reduced upon excitation of rhodopsin. Excited rhodopsin catalyzes the exchange of GTP for bound GDP on a GTP-binding protein (transducin or G);[1] this results in the formation of $G_{\alpha GTP}$ ($\alpha$ subunit of G with GTP bound) which is the activator of the rod cGMP phosphodiesterase (PDE) [reviewed by Pugh and Lamb (1990)]. Both G and PDE are peripheral proteins which can be easily dissociated from the membrane at low ionic strength and can reassociate to the membrane at moderate ionic strength (Kühn, 1985). Light-induced scattering changes in rod suspensions, first described by Hofmann et al. (1976) and by Bignetti et al. (1980), have been shown to be associated with the first steps of the light-induced enzymatic cascade: "P-signal" (Hofmann et al., 1976) or "binding signal" (Kühn et al., 1981) associated with the formation of a complex between excited rhodopsin and G in the absence of GTP; "dissociation signal" associated with the activation of G in the presence of GTP (Kühn et al., 1981) and, more precisely, with the dissociation of $G_{\alpha GTP}$ from the membrane (Bruckert et al., 1988). Several scattering changes were later described (Lewis et al., 1984; Caretta & Stein, 1985, 1986; Kamps et al., 1985; Bennett, 1986), which present a number of similarities, and in particular were all observed under conditions which correspond to the activation of PDE. The relation between these signals and PDE activation was, however, later questioned by results obtained with preparation of high structural integrity (Wagner et al., 1987).

We report here a further study of a light-scattering change (termed PAS for phosphodiesterase activation signal) which was previously shown to be specifically dependent on the presence of G, GTP, and PDE and in addition on a previous ATP-dependent process which was interpreted as swelling of the discs or vesicles (Bennett, 1986). In the present study, reconstituted systems of well-controlled protein and nucleotide composition were used. The results are compared to previous measurements of PDE activity under similar conditions (Bennett & Clerc, 1989).

## Experimental Procedures

Dark or bleached rod outer segments (ROS) are prepared from fresh bovine eyes according to the method of Kühn (1985) and kept in liquid nitrogen. ROS fragments composed

[1] Abbreviations: ROS, rod outer segment; R*, photoexcited rhodopsin; G, retinal G-protein (transducin); $G_{GDP}$ or $G_{GTP}$, G-protein with GDP or GTP bound; G*, activated G ($G_{GTP}$ or $G_{GTP\gamma S}$); GTP$\gamma$S, guanosine 5'-*O*-thiotriphosphate; PDE, cGMP phosphodiesterase.

0006-2960/92/0431-1858$03.00/0 