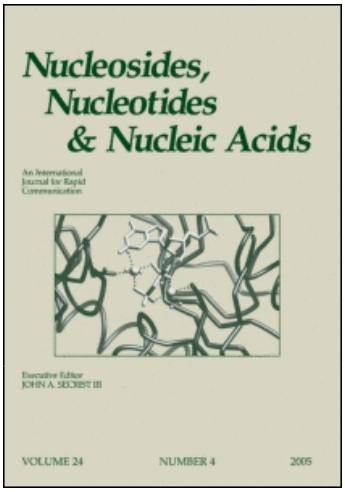

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UNIQUE ^{31}P SPECTRAL RESPONSE TO THE FORMATION OF A SPECIFIC RESTRICTION ENZYME–DNA COMPLEX

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□ *Protein-induced distortion is a dramatic but not universally observed feature of sequence-specific DNA interactions. This is illustrated by the crystal structures of restriction enzyme–DNA complexes: While some of these structures exhibit DNA distortion, others do not. Among the latter is* Pvu*II endonuclease, a small enzyme that is also amenable to NMR spectroscopic studies. Here ^{31}P NMR spectroscopy is applied to demonstrate the unique spectral response of DNA to sequence-specific protein interactions. The ^{31}P NMR spectrum of a noncognate DNA exhibits only spectral broadening upon the addition of enzyme. However, when enzyme is added to target DNA, a number of ^{31}P resonances shift dramatically. The magnitudes of the chemical shifts (2–3 ppm) are among the largest observed. Site-specific substitution with phosphoramidates and phosphorothioates are used analyze these effects. While such spectral features have been interpreted as indicative of DNA backbone distortions, FRET analysis indicates that this does not occur in* Pvu*II-cognate DNA complexes in solution. The distinct ^{31}P spectral signature observed for cognate DNA mirrors that observed for the enzyme, underscoring the unique features of cognate complex formation.*

Keywords ^{31}P NMR; DNA; Protein-DNA interactions; Restriction enzyme

INTRODUCTION

The mechanism of sequence-specific protein–nucleic acid recognition has long been among the richest problems in biochemistry. Coupled with the energetic basis of sequence discrimination,[1] structural details from x-ray crystallographic studies have provided valuable insights into this process. In addition to direct amino acid–base contacts, contacts between these proteins and the phosphodiester backbone are also observed.[2] As exemplified by the TBP/TATA box complex, some sequence-specific DNA-binding proteins induce obvious distortions in the nucleic acid backbone (i.e.,

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noncanonical phosphorus bond angles) that result in obvious nonlinearity or a bend in the duplex.[3] However, not all such crystal structures exhibit this behavior. The group of structures that best illustrates this dichotomy is type II restriction endonucleases, a class of homodimeric enzymes that exhibit exquisite sequence specificity and perform Mg(II)-dependent phosphodiester hydrolysis at their target (cognate) sites. In some cases, cognate DNA distorts significantly upon complexation with an enzyme.[4] In others, it is so much less pronounced it is afforded little discussion.[5,6]

These observations lead to speculation that either DNA distortion is not compulsory in cognate complexes and/or crystallography may not necessarily reflect solution behavior. The ideal system for examining this question is *Pvu*II endonuclease. This enzyme, which cleaves at 5′-CAGCTG-3′ at the central GC step to yield blunt ends,[7] induces no DNA distortion detectable by crystallography.[6] However, as the smallest of the well-characterized restriction enzymes (2 × 18 kD subunits), its complexes are amenable to NMR spectroscopy.[8–11] The high natural abundance and sensitivity of the ^{31}P nucleus provide a natural means of examining the DNA backbone responses to ligand binding in solution.[12–14] Interestingly, among the few protein–nucleic acid complexes studied with this technique, there is a strong correlation between highly shifted ^{31}P resonances in a cognate complex and crystallographically determined DNA distortion.[15–18] In this study, a combination of ^{31}P NMR spectroscopy and FRET analysis is used to establish the first case of highly ^{31}P shifted resonances that are not associated with protein-induced DNA distortion.

MATERIALS AND METHODS

Materials

Chelex resin was purchased from Biorad (Hercules, CA). Puratronic $MgCl_2$ and $CaCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). d-Tris was purchased from Cambridge Isotopes (Andover, MA). Concentrations of stock solutions were determined by flame atomic absorption spectroscopy using a Perkin Elmer AAnalyst 700 spectrophotometer. All buffers were applied to a Chelex column to remove adventitious metal ions. Subsequent pH adjustments were made with metal-free nitric acid. All solutions were determined by atomic absorption spectroscopy to be metal free to the limits of detection.[19]

Preparation of *Pvu*II Endonuclease

The recombinant expression system *Pvu*II endonuclease in *Escherichia coli*[20] were kindly provided by Dr. Paul Riggs of New England Biolabs. The cells were grown in minimal media (M9 salts) supplemented with

10 μg/mL biotin, 10 μg/mL uracil, 50 μg/mL thiamine, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.5% glucose. Purification of all enzymes was accomplished using phosphocellulose chromatography and heparin sepharose affinity chromatography as previously described.[8] Proteins were concentrated using Amicon Centriprep and Centricon concentrators and adventitious metal ions were removed via exhaustive dialysis against metal-free buffer.[21] In repeated determinations, metal ions in enzyme samples prepared in this fashion are undetectable (termed "metal-free"). All apoenzymes were quantitated using $\varepsilon_{280} = 36{,}900\ M^{-1}cm^{-1}$ for the monomer subunit and handled with metal-free sterile pipette tips and sterile plasticware to prevent contamination.

Preparation of Oligonucleotides

The following unmodified oligonucleotides were purchased from either the Biopolymer Synthesis Facility at the California Institute of Technology (Pasadena, CA) or IDT (Coralville, IA) and either obtained HPLC purified or subsequently purified via HPLC or gel electrophoresis: Self-complementary cognate 13-mer 5′-TGACCAGCTGGTC-3′; self-complementary noncognate 13-mer 5′-TGACCACGTGGTC-3′; top non-self-complementary cognate 14-mer strand 5′-TCCGCAGCTGCCTG-3′; and bottom cognate 14-mer strand 5′-CAGGCAGCTGCGGA-3′. The self-complementary 14-mer strand 5′-CGACCAGCTGGTCG-3′ containing the 3′-amidate linkage at the scissile phosphate was purchased HPLC purified from Transgenomic (Boulder, CO). 5′-fluorescein–labeled 14-mer top strand (5′-Fl-TCCGCAGCTGCCTG-3′) and 5′-rhodamine C6 amino 14-mer bottom strand (5′-Rh-CAGGCAGCTGCGGA-3′) were purchased from Midland Certified Reagent Company (Midland, TX) and gel purified.

DNA was quantitated using ε_{260} values provided by the vendor. All oligonucleotide concentrations are expressed with respect to the strand or duplex as indicated. Using Centricons, DNAs were rendered metal free through at least two exchanges of >90% volume with deionized distilled water. Subsequent handling was accomplished with metal-free pipette tips and sterile plasticware. Samples were stored in sterile water at 4°C for immediate use or lyophilized for storage.

NMR Analysis

One-dimensional ^{31}P NMR spectra were collected on a Bruker ARX 500 spectrometer at 202 MHz using a 5-mm direct detect broadband probe. NMR samples for one-dimensional ^{31}P NMR spectra contained at least 650 μM strands in 25 mM Tris, 200 mM KCl, pH 7.7 at 25°C. These high ionic strength conditions stabilize the enzyme for long, room temperature acquisitions.[8] Unless otherwise noted, enzyme was added in excess

(1.3 equiv.) to ensure complete complex formation. Native DNA spectra were collected with a sweep width of 12 ppm with 700 points; for phosphoramidate and phosphorothioate spectra, sweep widths were increased to 25 and 70 ppm, respectively, and the number of points increased proportionally. Typically, ^{31}P NMR spectra of free duplexes were collected with about 3000 scans. To ensure detection of poorly populated species, spectra of enzyme-DNA complexes were collected with at least 100,000 scans in 30% D_2O. ^{31}P chemical shifts are relative to external trimethylphosphate in D_2O.

Two-dimensional spectra of the free duplex were collected on the same spectrometer with a 5-mm inverse ^{1}H-broadband probe with a 1.6-mM strand sample in 25 mM d-Tris buffer, 200 mM KCl, 10 mM $CaCl_2$, pH 7.7 at 25°C in 100% D_2O. ^{1}H NOESY, COSY, and TOCSY data were collected at a sweep width of 11 ppm with 64 scans of 2048 points each. For the NOESY spectrum, the mixing time was 150 ms. ^{1}H-^{31}P COSY spectra were collected with a sweep width of 3.4 ppm, 128 scans, and 512 points in the ^{1}H dimension; the sweep width was 1.5 ppm with 256 points in the ^{31}P dimension. For all ^{1}H experiments, solvent suppression was accomplished using presaturation, and spectra were referenced to DSS.

Fluorescence Spectroscopy

Fluorescence emission spectra were collected on a Spex Fluorolog-3 spectrofluorimeter. The temperature was maintained with a thermostated compartment at 25°C. All samples were monitored with stirring using a nitric acid–cleaned quartz cuvette. The sample was excited at 485 nm and intensity recorded from 500 to 650 nm. The bandpasses were set to 1 nm. Strands were titrated with one another to correct for any errors in quantitation. To ensure quantitative complex formation, experiments were conducted on 65 nM strands in the absence and presence of 600 nM enzyme dimers in 25 mM Tris, 200 mM KCl, 10 mM $CaCl_2$ at pH 7.5, conditions for which we have extensive binding data.[22] Enzyme-DNA complex formation was confirmed by anisotropy during the experiment.[22]

Graphics

Distances, torsion angles, and bond angles were measured in the indicated files using Swiss PDB Viewer 3.7.

RESULTS

DNA Spectral Responses and Sequence Specificity

The *Pvu*II enzyme conformational response to cognate complex formation is unique; more specifically, ^{1}H-^{15}N HSQC spectral patterns observed

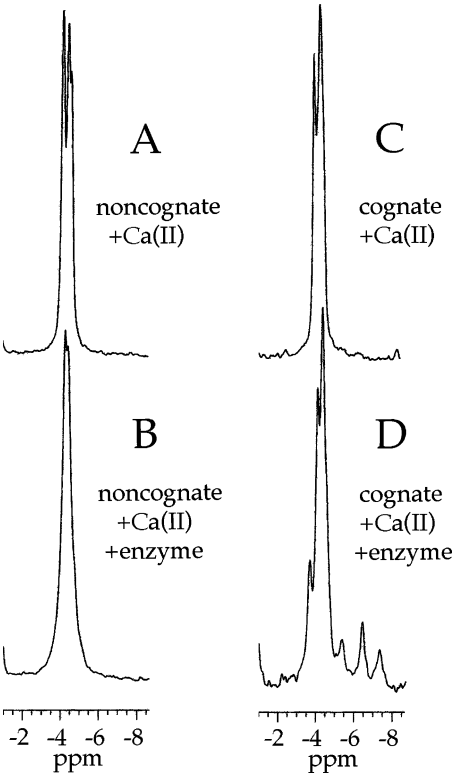

FIGURE 1 ^{1}H-decoupled ^{31}P NMR spectra of self-complementary noncognate DNA in the absence (A) and presence of enzyme (B). Self-complementary cognate 13-mer duplexes in the absence (C) and in the presence of enzyme (D). Conditions: 0.65-mM 13-mer strands, at least 1.3 equiv. of *Pvu*II endonuclease monomers as indicated, 10 mM $CaCl_2$, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, 25°C. Free duplex spectra were collected with 3000 transients. To ensure detection of poorly populated species, spectra of enzyme-bound duplex samples required at least 100,000 transients. FIDs of 700 points were processed with zero filling and line broadening. Chemical shifts are relative to external trimethylphosphate. See text for detailed interpretation.

for the *Pvu*II enzyme in a cognate complex are dramatically distinct from those in a noncognate complex.[11] It is therefore reasonable to hypothesize that DNA mirrors this behavior.

To assess a general ^{31}P spectral response of DNA to complex formation, spectra were collected of a noncognate duplex DNA in the absence and presence of enzyme and the DNA binding cofactor Ca(II). The ^{31}P NMR spectrum of a free noncognate oligonucleotide duplex appears in Figure 1A. The narrow range of resonances between−4 and−5 ppm is typical for B-form DNA.[23] Upon the addition of enzyme, spectral broadening is evident (Figure 1B). This is consistent with the formation of an intermolecular complex (40 kD) in either slow or intermediate exchange. There appears to be no dramatic changes in ^{31}P chemical shift associated with this complex.

In contrast, a much different spectrum is exhibited by cognate DNA bound by *Pvu*II endonuclease (Figures 1C and 1D). In addition to the expected spectral broadening, a number of dramatically upfield-shifted ^{31}P resonances are visible at −5.4, −6.5, and −7.4 ppm.

Identification of Enzyme-Shifted Resonances

To determine if there is a correlation between the shifted resonances and known structural features of the complex, resonance assignments were undertaken. In small, well-behaved complexes, one can take advantage of fast exchange kinetics to correlate free and bound resonances[15] and/or utilize ^{1}H-^{31}P correlation experiments.[17] However, due to the influence of particle size on T_2,[24] such experiments require linewidths that are only achievable in small complexes.

An alternative approach to assignments which can be applied to larger complexes is to incorporate substitutions which would alter the spectrum in a specific manner. The replacement of a nonbridging oxygen with sulfur results in a large downfield ^{31}P chemical shift of that phosphate. Indeed, phosphorothioates resonate around 52 ppm (Figure 2A).[23] Ideally, the resonance of the phosphate containing sulfur would be missing from the native phosphodiester region of the spectrum (Figure 2B) and thus identified. Of course, the introduction of sulfur at phosphates participating in key contacts with the enzyme can compromise complex stability.[25] However, by increasing the number of scans, even the weakest complexes should be sufficiently populated at millimolar concentrations to produce informative spectra. Other potential complications are collateral perturbations, i.e., substitution at one location causes chemical shift changes elsewhere in the strand or duplex. Provided these are not dramatic, this technique should be useful in the assignment of resonances.

Assignment efforts began by collecting spectra of enzyme-bound duplexes containing sulfur on only one strand. In each case, the highly shifted peaks were preserved, but one peak was reduced in intensity (data not shown). This is consistent with the perturbation of a remaining native linkage, most likely on the opposing strand. To confirm this, spectra were collected of complexes that feature phosphorothioate linkages directly *across* from one another in the duplex; i.e., a monothioate substitution at the scissile (5′-TGACCAG*CTGGTC-3′) and dithioate substitutions at nonscissile linkages 5′-TGACCA*GC*TGGTC-3′, 5′-TGACC*AGCT*GGTC-3′, and 5′-TGAC*CAGCTG*GTC-3′. Since the phosphorothioates are synthesized as diastereomers, the complexes are mixtures thereof (Figure 2A, inset). As shown in Figure 2, spectra of these oligonucleotides in the presence of enzyme proved to be quite informative. The sizes of the shifted peaks relative to those of the unmodified phosphates indeed vary, consistent with weakened binding in some cases. This phenomenon is especially obvious in

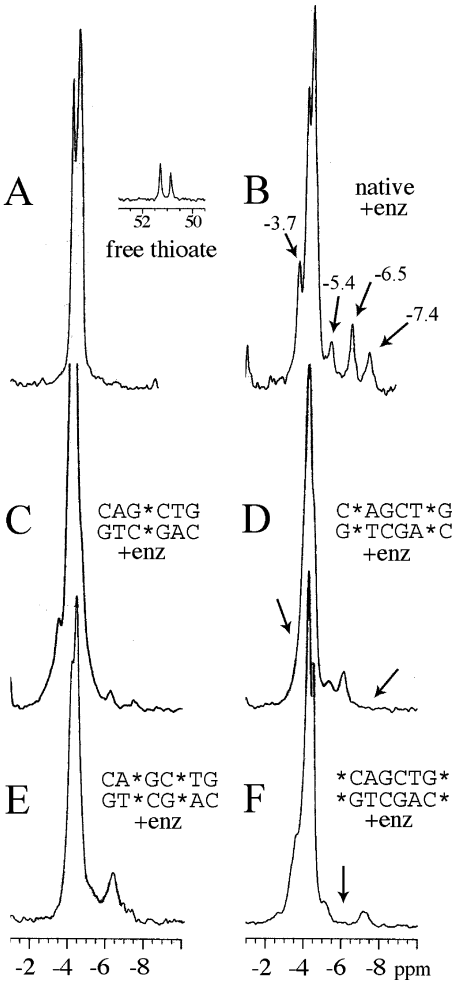

FIGURE 2 ^{1}H-decoupled ^{31}P NMR spectra of self-complementary mono- (A,C) and di- (D-F) phosphorothioate substituted 13-mer duplexes in the presence of *Pvu*II endonuclease. The inset in (A) features the diastereomeric signals of a single phosphorothioate center. The positions of phosphorothioate substitution are indicated with stars. Arrows denote the locations of resonances lost due to phosphorothioate substitution. Conditions: At least 0.65-mM strands, at least 1.3 equiv. enzyme monomers, 10 mM $CaCl_2$, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, 25°C. Free duplex spectra were collected with 3000 transients. To ensure detection of poorly populated species, spectra of enzyme-bound duplex samples required at least 100,000 transients. FIDs of 4284 points were processed with zero filling and line broadening. Chemical shifts are relative to external trimethylphosphate. See text for detailed interpretation.

the spectrum of the complex in which the phosphorothioate is located at the scissile phosphate (5′-TGACCAG*CTGGTC-3′; Figure 2C). Although there are some differences in intensity and linewidth, the peak at −7.4 ppm is present in all spectra except that of 5′-TGACC*AGCT*GGTC-3′ (Figure 2D). Similarly, the peak at −6.5 ppm is notably absent in the spectrum of 5′-TGAC*CAGCTG*GTC-3′ (Figure 2F). While it cannot be ruled out that

peaks have shifted due to collateral effects, the missing peaks are assigned to the linkages modified in those samples and thus to the corresponding linkages in the native substrate. Since symmetrical sulfur substitution on both strands was required to eliminate shifted peaks (see above), sets of two linkages were assigned (C_5pA_6/T_9pG_{10} and $C_4pC_5/G_{10}pG_{11}$, respectively).

There are two other relatively well-resolved resonances that merit discussion. The first is the peak at −3.7 ppm, on the downfield shoulder of the central intensities. In spite of some slight shifting and broadening, it is present in all spectra except Figure 2D and E. In these spectra, the range of resonances is narrower than in the other spectra, consistent with the disappearance of this peak. If one argues that the disappearance of this peak in Figure 2D is a collateral shift (since the missing peak is assigned to C_5pA_6/T_9pG_{10}), then the peak at −3.7 ppm could be tentatively assigned to A_6pG_7/C_8pT_9. The remaining peaks of interest, the one at −5.4 ppm and the one corresponding to the scissile phosphodiester linkage, could not be assigned.

Free Oligonucleotide Resonance Assignments

In order to gauge the extent of chemical shift changes, a series of two-dimensional reference spectra were collected of the free duplex. A ^1H NOESY spectrum was used to observe through-space sequential connectivities between base and sugar protons that are characteristic of duplex DNA (principally H6 and H8 base protons to H1′ and H2′2″ sugar protons).[24] A number of other base-to-sugar proton connectivities were also observed in this spectrum (H6 and H8 to H3′, H4′ and H5′5″). Assignments were clarified using ^1H COSY and ^1H TOCSY spectra, which correlate neighboring protons and those of entire ribose spin systems, respectively. This permitted the assignment of H3′ and H4′ sugar protons, which are in turn coupled to ^{31}P nuclei of the phosphate backbone. Finally, the resonances of these sugar protons were correlated to ^{31}P resonances with a ^1H-^{31}P COSY experiment[17] (Figure 3). In this fashion, a number of signals in the one-dimensional ^{31}P spectrum of the native free duplex were assigned. Due to spectral overlap in a number of base chemical shifts (e.g., T_9 and T_{12}, G_{10} and G_7), a few sugar proton assignments were rendered ambiguous, leaving a few phosphates unassigned (data not shown).

In those cases where both free and bound chemical shifts are known, $\Delta\delta$ is easily calculated. In instances in which free, bound, or both chemical shifts are ambiguous, the width of the central cluster of intensities was used to establish limits of the chemical shift change (−4.10 to −4.64 ppm for free and −3.7 to −5.4 ppm for bound). Chemical shift assignments are summarized in Figure 4. Interestingly, resonances corresponding to linkages at or near the scissile phosphate shift less dramatically than outer linkages.

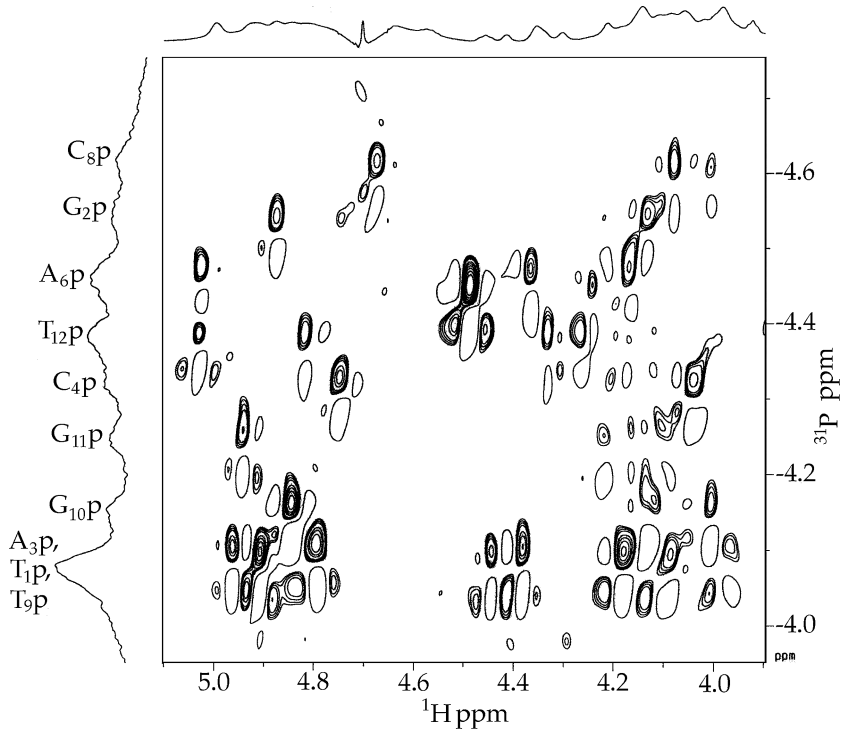

FIGURE 3 ^{1}H-^{31}P COSY spectrum of 5′$T_1G_2A_3C_4\underline{C_5A_6G_7C_8T_9G_{10}}G_{11}T_{12}C_{13}$-3′. Negative intensity is represented by one contour; positive intensities have multiple contours. Assignments of phosphodiester linkages were achieved by correlating ^{31}P-coupled ^{1}H signals to base protons in ^{1}H NOESY spectra as described in the text. Due to ambiguities with sugar proton assignments arising from spectral overlap, assignments of C_5p and G_7p were not made. Conditions: 1.6-mM strands, 25 mM Tris, 200 mM KCl, 10 mM $CaCl_2$, 100% D_2O, pH∗ 7.7, 25°C. FIDs of 512 points from 128 scans were processed with zero filling and Gaussian multiplication (line broadening −8 Hz). Chemical shifts are relative to external trimethylphosphate.

These latter linkages exhibit some of the largest ^{31}P chemical shift changes reported for a protein-bound oligonucleotide.[15,17]

Assay of DNA Bending by Energy Transfer

Dramatic changes in ^{31}P chemical shift (>1 ppm) in protein-DNA complexes are often interpreted as evidence for DNA "distortion"[15–17] (see Discussion). Here, the term "distortion" refers to local noncanonical perturbations in phosphorus bond angles to which ^{31}P chemical shift is sensitive.[12] The term "bending" is often used synonymously but actually refers to visually obvious nonlinearity of the duplex that results from local DNA distortions.

The intriguing thing about the *Pvu*II system is that ^{31}P chemical shift changes typically associated with distortions are observed in a complex for

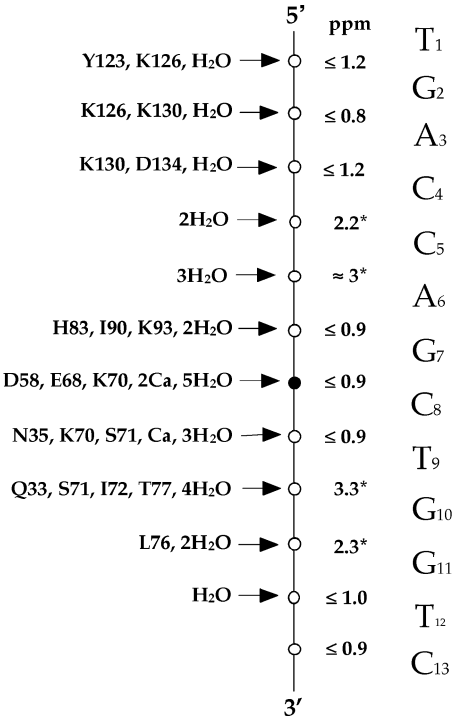

FIGURE 4 Summary of chemical shift changes upon *Pvu*II endonuclease binding. The DNA is a self-complementary 13-mer. Phosphodiester linkages are indicated by the circles; the filled circle represents the scissile phosphodiester linkage. Intermolecular contacts within 5 Å of phosphorus observed in at least one subunit by x-ray crystallography (1F0O.pdb) are indicated with arrows. Starred chemical shifts are known to be upfield shifts; in all other cases the direction of the shift is unknown. Limits in $\Delta\delta$ were determined using the chemical shift ranges for the free and enzyme-bound resonance envelopes as described in the text. Adapted from Cheng et al. [6]

which little to no DNA distortion is observed crystallographically.[6,26] This suggests two possibilities: One, that *Pvu*II endonuclease distorts DNA in solution, but this behavior is not accessed in crystal form; two, the enzyme does not distort DNA, and the observed ^{31}P chemical shift changes are due to other effects (see Discussion).

To distinguish between these two possibilities, DNA bending assays were performed using fluorescence resonance energy transfer (FRET) methodology.[27,28] Briefly, the effect of enzyme binding on the efficiency of energy transfer between a fluorescein-rhodamine donor-acceptor pair in a non-self-complementary 14-mer cognate duplex is measured. Enzyme-induced DNA bending will result in a decrease in the donor–acceptor distance; this increases the efficiency of energy transfer and is reflected in a decrease in donor emission in the presence of the acceptor. This provides a means of assessing broad-range effects of DNA distortion.

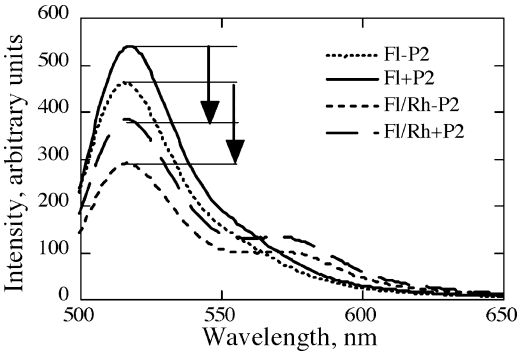

FIGURE 5 FRET assay of DNA bending. 14-mer Duplex labeled in the top strand with fluorescein in the absence (dotted) and presence (solid) of enzyme. The enzyme causes an increase in fluorescein emission. A decrease in fluorescein emission is observed with free duplex labeled with both fluorescein and rhodamine (short dash). The addition of enzyme to the donor/acceptor labeled duplex in the presence of Ca(II) results in an increase in fluorescein emission (long dash). Since no net decrease in donor emission occurs upon the addition of enzyme (compare the length of the arrows), DNA bending is not appreciable. Conditions: 65-nM duplex, 600-nM enzyme dimers (where indicated) in 25 mM Tris, 200 mM KCl, 10 mM $CaCl_2$, pH 7.5, 25°C.

In a FRET assay of DNA bending, it is critical to distinguish contributions from energy transfer from other effects. Given the proximity of the dyes to the enzyme in the complex, it is reasonable to expect enzyme binding to alter emission properties independent of bending effects. To characterize this behavior, emission spectra of a duplex labeled only with fluorescein on the top strand were collected in the absence and presence of enzyme. As illustrated in Figure 5, enzyme binding causes an increase in fluorescein emission intensity (dotted to solid curve).[28] As expected, fluorescein emission decreases when the free duplex is formed with the rhodamine-labeled bottom strand, indicating energy transfer (compare dotted- to short-dashed curve). Upon the addition of enzyme to the donor/acceptor labeled duplex in the presence of Ca(II), an increase in fluorescein emission is observed (compare short to long dashed curves). If it is assumed that the increase in fluorescein donor emission induced by the enzyme is the same in both the presence and absence of the rhodamine donor,[28] then no change in energy transfer efficiency occurs upon the addition of enzyme (compare the length of the arrows). This is in contrast to that expected from bending; i.e., a decrease in donor emission relative to the free labeled duplex.

Effects of Metal Ion Cofactors

A number of restriction enzymes require a divalent metal ion cofactor for high-affinity cognate DNA binding. In many studies this is accomplished with Ca(II), which promotes DNA binding but not cleavage.[29–31]

While *Pvu*II endonuclease exhibits a characteristic enzyme conformational response to cognate DNA binding in the absence of metal ions,[11] thermodynamically it cannot discriminate between cognate and noncognate sites unless this cofactor is present.[22]

To determine if Ca(II) is required to achieve the ^{31}P NMR spectrum characteristic of *Pvu*II:cognate DNA complex observed above, ^{31}P NMR spectra of cognate DNA-*Pvu*II endonuclease complexes were also collected in the absence of metal ions and in the presence of the native cofactor Mg(II). To prevent DNA cleavage in the latter instance, phosphoramidates (PN) were used as substrate analogues. These DNAs, in which the 3′ oxygen of the scissile phosphate is replaced with nitrogen, are stable in the presence of *Pvu*II endonuclease and Mg(II) and bind the enzyme with comparable affinity.[10]

^{31}P NMR spectra of the enzyme:cognate phosphoramidate complex in the presence of Mg(II) and under metal-free conditions were collected and compared to that collected in the presence of Ca(II) (Figure 6). The peaks at 3.3 ppm are ascribed to isomers of the phosphoramidate linkage,[10] and the intensity near 0 ppm is attributed to residual phosphate buffer from enzyme purification. In addition to the typical resonances between −4 and −5 ppm, spectra collected in the presence of Mg(II), Ca(II), or in the absence of added metal ion all exhibit significantly shifted phosphate peaks between −5 and −8 ppm (Figures 6B, C, and D). From these spectra it appears that metal ions are not needed to induce phosphate backbone chemical shift changes that are characteristic of cognate complexes, a result that is consistent with a number of previous restriction enzyme studies.[11,29–31]

DISCUSSION

DNA Conformation in Restriction Enzyme Complexes

Historically, the structural biology of restriction enzymes has focused on specific amino acid–base contacts and conformational adjustments in the enzyme.[4] The effect of restriction enzyme binding on DNA sequences, both cognate and otherwise, has received less attention. Early DNA footprinting studies of *Eco*RI endonuclease revealed hypersensitive sites, which are usually interpreted as indicative of DNA distortions.[32] Indeed, the *Eco*RI-DNA crystal structure exhibits DNA distortions that have been described as neokinks.[33] Recently, a more complex picture has emerged from crystallographic studies. Restriction enzyme–DNA crystal structures appear to vary substantially in the degree of distortion observed in the phosphodiester backbone. For *Eco*RV endonuclease, enzyme-induced DNA bending of the cognate sequence is quite pronounced (50° kink).[34] Although less dramatic, this is also observed for the *Mun*I enzyme.[35] *Bgl*I endonuclease induces a less dramatic but nevertheless perceptible bend in

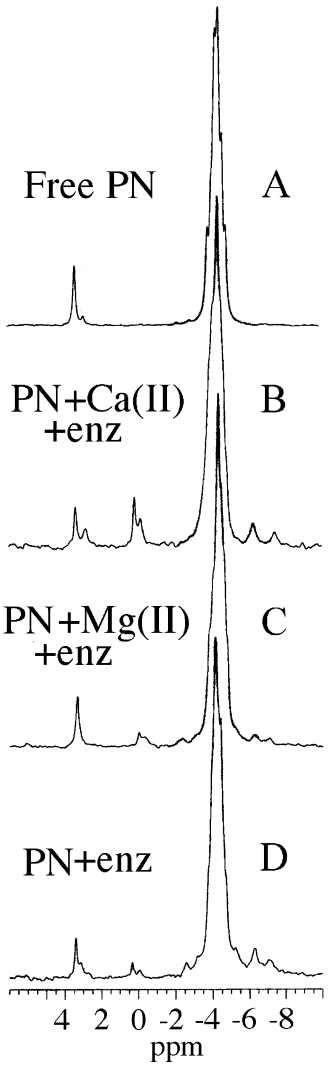

FIGURE 6 ^{1}H-decoupled ^{31}P NMR spectra of cognate self-complementary phosphoramidate (PN) duplexes. Free phosphoramidate (A), PN in the presence of Ca(II) and enzyme (B), in the presence of Mg(II) and enzyme (C), and in the presence of enzyme but in the absence of added metal ions (D). Spectra in (A) and (B) have been adapted from King et al.[10] Conditions: 0.65-mM enzyme monomers, 25 mM Tris, 200 mM KCl, 10 mM metal ion as indicated, 10% D_2O, pH 7.7, 25°C. Because samples were initially prepared for HSQC experiments, they contain excess 14-mer PN. FIDs of 700 points from 175,000 scans were processed with zero filling and line broadening. Chemical shifts are relative to external trimethylphosphate. Intensity near -1 ppm is attributed to residual phosphate buffer from enzyme purification. See text for detailed interpretation.

the DNA (20°).[36] In sharp contrast, *Bam*HI[5] and *Bso*BI[37] endonucleases produce only slight deviations from canonical B form DNA. There are no significant perturbations in DNA structure in the *Pvu*II endonuclease complex crystal structure.[6]

^{31}P NMR Spectroscopy of Protein–Nucleic Acid Complexes

To put the results presented here into perspective, it is useful to consider ^{31}P NMR spectra of the few other protein–nucleic acid systems so characterized. In two separate ^{31}P NMR studies of the DNA complexes of the HMG box protein SRY, dramatic shifting of multiple ^{31}P resonances was observed (up to 1.3 ppm in both directions).[17,38] Indeed, the solution structure of this complex reveals that SRY bends DNA 70°.[39] This is supported by alkylation interference experiments, the results of which necessitate a dramatic bend.[38]

Another well-characterized system is the *lac* repressor headpiece. Upon headpiece binding, phosphate groups of the WT operator O1 exhibit shifts up to 0.2 ppm; a similar pattern is observed with O2, with shifts up to 0.4 ppm. Perhaps due to the use of a protein fragment, chemical shift changes are modest and smaller than what is observed here. Most of the movements are upfield and are attributed primarily to conformational adjustments in the phosphodiester linkages due to headpiece binding. The crystal structure cognate DNA complexed with an intact *lac* dimer features a DNA bend of 45°.[40] It is unclear whether or not more dramatic changes in ^{31}P chemical shift would be observed in the presence of intact protein, if such spectra would be achievable.

The *trp* repressor system has also been characterized by ^{31}P NMR spectroscopy and x-ray crystallography. The ^{31}P NMR spectrum of this complex is characterized by a number of dramatically upfield shifted peaks (up to at least 2 ppm).[16] Crystallographers have characterized a 20° bend in the 1:1 operator complex.[2] Interestingly, there is no severe localized distortion; rather, the bend is attributed to the cumulative effects of modest distortions across a broad contact area (18 bp).

The *Pvu*II system shares with these examples dramatic ^{31}P chemical shift changes upon protein binding. However, unlike these systems, no significant distortions in the DNA are observed by x-ray crystallography.[6,26] This is supported by the energy transfer experiments described herein. Since distortions are the most common interpretation of shifted ^{31}P resonances, this presents an interpretive challenge. Led by work published by Gorenstein and collaborators, it is generally agreed that changes in torsion angles (α, O3′-P-O5′-C5′; ζ, C3′-O3′-P-O5′) and bond angles (OPO) are major determinants of ^{31}P chemical shift changes.[12] The most dramatic examples of geometric changes are those induced by intercalators. Downfield shifts have been observed repeatedly in the ^{31}P NMR spectra of drug-bound DNAs[13,14] and also in SRY, which intercalates an Ile into the base stack.[39] However, more subtle effects can also be reflected in ^{31}P NMR spectra: Based on work with model compounds, small changes in bond angle affect large changes in ^{31}P chemical shift.[12] This would suggest that even slight changes in geometry that would not look especially dramatic when viewing

a structure could indeed cause a significant change in chemical shift. However, examination of two *Pvu*II-DNA crystal structures (1F0O.pdb and 1PVI.pdb) reveal no correlation between the large changes in chemical shift that are observed for the noted linkages and the small deviations in OPO bond angles or α and ζ torsion angles.

There is some indication that other factors may significantly influence ^{31}P chemical shifts. In a study of NADPH-dihydrofolate reductase interactions, the authors argue that shielding effects in the form of hydrogen bonds (rather than changes in torsion angles) dominate the behavior of ^{31}P NMR chemical shifts of pyrophosphate nuclei.[41] In the ^{31}P NMR study of the *trp* repressor-operator interactions, salt bridges are also suggested as possible influences on ^{31}P chemical shifts in protein-DNA complexes.[16]

This prompted an examination of crystal structures for interactions that could be used to rationalize chemical shift perturbations. Indeed, the *trp* repressor-operator structure is well known for the multitude of contacts between the protein and the DNA backbone.[2] The *Pvu*II complex structure is similar: As summarized in Figure 4, several contacts between protein moieties and phosphodiester linkages have been characterized by x-ray crystallography (1F0O.pdb).[26] A couple of the assigned linkages for which there are large ^{31}P chemical shift changes do indeed make contacts with the protein (T_9pG_{10} with Q33 and $G_{10}pG_{11}$ with L76). However, the enzyme also directly interacts with a similar number of linkages that shift less significantly (T_1pG_2 with K126 and Y123; G_2pA_3 with K126, K130, and D134; A_3pC_4 with K130 and K137; A_6pG_7 with H83, I90, and K93; G_7pC_8 with D58, E68, and K70; and C_8pT_9 with N35, K70, and S71). Further, two of the highly shifted phosphates (C_4pC_5 and C_5pA_6) are highly solvated, exhibiting no direct protein contacts within 5 Å of the phosphorus atom.

These kinds of observations are consistent with multiple influences on the strong chemical shift anisotropy exhibited by the ^{31}P nucleus. By definition, this phenomenon can be very directional; that is, two or more shielding tensors can be affected in different ways.[12] It is reasonable to speculate that multiple directional effects combine in a unique way in the cognate complex, giving rise to the large chemical shift changes observed. It may also explain why the correlation between $\Delta\delta$ and the degree of bending observed in the systems described is not directly proportional.

Conformational Uniqueness of Cognate Complexes

Regardless of the origins of the changes in ^{31}P chemical shifts observed with target DNA, they are unique to the cognate complex. These features are *not* observed in the presence of noncognate DNA. Similar patterns have been observed in the *trp* repressor system. DNA affinity of the *trp* aporepressor is generally weak, nonspecific, and ^{31}P NMR spectra of *trp* repressor-operator complexes do not exhibit dramatic changes in chemical

shift. However, when Trp is added, a specific complex is formed, and dramatic shifts in the ^{31}P spectrum are observed.[16]

In the *lac* system, ^{31}P spectra of mutant operators O3 and O4 in the presence of headpiece protein were characterized as exchange broadened and feature no dramatic chemical shift changes. The authors rationalize this as multiple binding events on the operator fragment. This is easier to visualize in the *lac* repressor headpiece system than in the *Pvu*II system: While both studies utilize oligonucleotides of the same size (14 bp), the headpiece is only 56 residues in length; a *Pvu*II monomer has 157 residues. In crystal structures, there is very little oligonucleotide surface that does not contact the enzyme; this suggests that multiple binding modes on such a small substrate are unlikely for *Pvu*II endonuclease.

Thus it appears that ^{31}P spectral differences are indicative of unique features which distinguish cognate from noncognate binding processes, and the data presented here provides an important counterexample that demonstrates that these features do not necessarily reflect significant DNA distortions.

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