Binding and Conformational Analysis of Phosphoramidate−Restriction Enzyme Interactions†

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ABSTRACT: Phosphoramidates are modified deoxyoligonucleotides that feature nitrogen in place of the 3′-oxygen of a phosphodiester linkage. Noted for stability against nuclease activity, these linkages are of both mechanistic and therapeutic interest. While a number of studies characterizing the properties of oligonucleotides composed entirely of phosphoramidate linkages have been published, little is known about how singly substituted phosphoramidate substitutions affect the thermodynamics and structure of protein−oligonucleotide interactions. We chose to investigate these interactions with *Pvu*II endonuclease, the DNA binding behavior of which is well-characterized. Oligonucleotide duplexes containing a phosphoramidate substitution at the scissile phosphates were resistant to cleavage by the enzyme, even after extended incubations. However, the enzyme was able to cleave the native strand in a native:phosphoramidate heteroduplex at a rate comparable to that observed with the native substrate. Ca(II)-stimulated *Pvu*II binding for a phosphoramidate-substituted oligonucleotide is comparable to that of the native duplex ($K_d \approx 200$ pM). K_d values obtained in the presence of Mg(II) are somewhat weaker ($K_d \approx 10$ nM). Under metal-free conditions, the enzyme exhibited a remarkable ≈50-fold greater affinity for the modified oligonucleotide relative to the native substrate (5 vs 240 nM). While ^{31}P NMR spectra indicate increased chemical shift dispersion in the free phosphoramidate duplex, the spectrum of the enzyme-bound duplex is similar to that of the native duplex. ^{1}H−^{15}N HSQC analysis indicates that enzyme conformations in the presence of these oligonucleotides are also comparable. The tight binding of the phosphoramidate duplex under metal-free conditions and its resistance to cleavage are attributed to local conformational adjustments propagating from the O→N substitution.

Antisense technology, in which nucleic acids are used to target their complements, relies on the resistance of such agents to the nuclease activities present in cells. Toward this end, a variety of nucleic acids that feature modified phosphodiester backbones have been designed and synthesized (*1*). One promising modification is found in N3′→P5′ phosphoramidates, in which the 3′-phosphate bridging oxygen is replaced by nitrogen (Figure 1). Oligonucleotides composed entirely of these linkages retain polyanionic character, are stable under basic and neutral conditions, and form Watson−Crick duplexes, which are slightly more stable than their native counterparts (*2*). Most importantly, under both in vivo and in vitro conditions, they are resistant to a variety of nucleases (*3*−*5*).

Most of the information available about the properties of phosphoramidates has emerged from studies of oligonucleotides featuring uniformly modified phosphodiester linkages. NMR and circular dichroism spectroscopic studies indicate that these oligonucleotides adopt an A-like conformation, typified by a C3′ endo sugar pucker (*6*−*9*). Information about interactions with sequence specific nucleases, which target a single phosphodiester linkage in an oligonucleotide duplex, is more scant. Early pilot studies by Shabarova and colleagues indicate that phosphoramidates are resistant to sequence specific nucleases (*4*, *10*), but little quantitative data have been published.

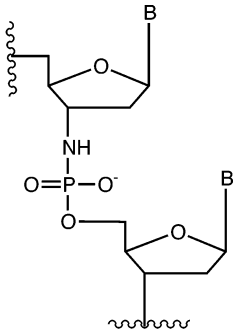

FIGURE 1: Structure of the N3′→P5′ phosphoramidate linkage in DNA.

Phosphoramidates are of special interest for students of nuclease mechanism. Behavioral differences relative to native oligonucleotides can inform our understanding of the reactivity and conformational subtleties that are critical to enzyme activity. In addition, well-characterized phosphoramidates can serve as valuable nonhydrolyzable substrate analogues with which to conduct structure−function studies.

Type II restriction enzymes recognize palindromic sequences of 4−8 base pairs and promote cleavage of double-stranded DNA. Paradigms for combining sequence specific DNA recognition with Mg(II)-dependent phosphodiester cleavage, they have been the subject of a wide variety of crystallographic and mechanistic studies (*11*, *12*). For the past several years, we have been focusing on *Pvu*II endonuclease, a homodimeric enzyme that recognizes the sequence 5′-CAG|CTG-3′ and cleaves it at the central base

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Table 1: Oligonucleotide Duplexes Used in This Study[a]

name	description	oligonucleotide duplex
O-NSC16	native nonself-complementary 16-mer	5′-TGTAGGCAGCTGCGGAG-3′ 3′-CATCCGTCGACGCCTC-5′
O-SC14	native self-complementary 14-mer	5′-CGACCAGCTGGTCG-3′ 3′-GCTGGTCGACCAGC-5′
N-NSC16	phosphoramidate nonself-complementary 16-mer	5′-TGTAGGCAG*n*CTGCGGAG-3′ 3′-CATCCGTC*n*GACGCCTC-5′
N-SC14	phosphoramidate self-complementary 14-mer	5′-CGACCAG*n*CTGGTCG-3′ 3′-GCTGGTC*n*GACCAGC-5′

[a] End HEX-labeled oligonucleotides were used as indicated in the text. The *Pvu*II recognition sequence is underlined. *n* indicates the location of the phosphoramidate modification.

step to achieve blunt ends. We have conducted quantitative studies that describe both metal ion-dependent and metal ion-independent DNA binding of this enzyme (*13*), and we have developed NMR spectroscopy as a probe of restriction enzyme conformation in solution (*14*−*16*). This level of characterization makes this enzyme an ideal system for examining protein−phosphoramidate interactions. How resistant is a phosphoramidate scissile linkage to cleavage? How does this linkage affect both metal ion-dependent and metal ion-independent DNA binding by a restriction enzyme? Are there significant conformational consequences to the introduction of this linkage at the scissile phosphate? Here, we address these issues using a combination of kinetic, equilibrium, and spectroscopic methods.

MATERIALS AND METHODS

Materials. Nitrocellulose filters (0.2 μm pore size) were purchased from Schleicher and Schuell (Keene, NH). Chelex resin was purchased from Biorad (Hercules, CA). Puratronic $MgCl_2$ and $CaCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). All buffers were rendered metal-free using Chelex resin prepared as described by the vendor and verified by atomic absorption spectroscopy (*17*).

Quantitation of Metal Ion Stocks. Concentrations of Mg(II), Mn(II), and Ca(II) stock solutions were determined by flame atomic absorption spectroscopy using a GBC model 904BT double beam atomic absorption spectrophotometer.

Preparation of PvuII Endonuclease. The recombinant *Escherichia coli* expression system for wild-type *Pvu*II endonuclease was 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. Uniform incorporation of ^{15}N was accomplished by providing $^{15}NH_4Cl$ as the only nitrogen source, save 40 μg/mL of Met required for growth. The purification of the enzyme was accomplished using phosphocellulose chromatography and heparin sepharose affinity chromatography as previously described (*14*). The proteins were concentrated using Amicon Centriprep and Centricon concentrators and rendered metal-free via exhaustive dialysis against metal-free buffer (*18*). All enzymes were quantitated using $\epsilon_{280} = 36\,900\ M^{-1}\ cm^{-1}$ for the monomer subunit and subsequently expressed with respect to the dimer.

Preparation of Oligonucleotides. Table 1 summarizes the oligonucleotide duplexes used in this study. Native unlabeled and 5′-hexachlorofluorescein (HEX)-labeled oligonucleotides were purchased desalted from IDT Technologies (Coralville, Iowa) and purified by polyacrylamide gel electrophoresis (PAGE) and Elutrap (Schleicher and Schuell). Oligonucleotides containing the 3′-phosphoramidate linkage at the scissile phosphate were purchased high-performance liquid chromatography (HPLC) purified from Transgenomic (Boulder, CO) and rendered salt and metal-free by rinsing repeatedly using Centricon concentrators. The DNAs were quantitated using ϵ_{260} values provided by the vendor. All oligonucleotide concentrations are expressed with respect to the duplex. Duplexes were formed by heating to 95 °C a mixture of 1 equiv of one strand with 1 equiv of the complementary strand and permitting the sample to cool to room temperature overnight. The samples were stored in sterile water at 4 °C for immediate use or lyophilized for storage.

Where appropriate, 17 pmol of duplex DNA was radiolabeled using ^{32}P-γATP (33 pmols of a 6000 Ci/mmol stock) (Perkin-Elmer, Boston, MA) and polynucleotide kinase (1 unit) as per the manufacturer's instructions (New England Biolabs, Beverly, MA). Following incubation for 2 h at 37 °C, the duplex was purified using Sephadex G-50 resin (Sigma, St. Louis, MO).

Nitrocellulose Filter Binding Assays. The filters were soaked in binding buffer immediately prior to use, backed with multiple layers of soaked filter paper, and inserted into a Slot-Blot apparatus (Biorad). Arrays of 500 μL binding reactions containing fixed concentrations of radiolabeled duplex and varying concentrations of enzyme were assembled in 96 well microtiter plates and incubated as appropriate at 25 °C. The samples were then vacuum filtered through the Slot-Blot apparatus and washed with ≈200 μL of binding buffer and dried. The filters were then wrapped in Saran Wrap and exposed to a phosphorimager screen overnight. The resulting image was scanned on a Storm Phosphorimager, and the digitized image was analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Areas of intensity were selected using the ImageQuant box tool. For consistency, the boxes for all selected intensities for a particular image were kept the same size. Following background correction, the intensities were transferred into a Microsoft Excel file and normalized. The corrected intensity is defined as the signal intensity of each protein concentration minus the signal intensity where no protein is added. This value is then divided by the maximum signal intensity for a particular protein series.

Assays of PvuII Endonuclease Activity. The hydrolysis activity of *Pvu*II endonuclease was assessed discontinuously

by denaturing PAGE analysis. Unless otherwise noted, 125 nM ^{32}P 5′-end-labeled duplex DNA was incubated with 625 nM *Pvu*II endonuclease dimers in 50 mM Tris, 100 mM NaCl, and 10 mM $MgCl_2$ at pH 7.5 and 37 °C. Each reaction was initiated by the addition of enzyme. At the indicated time, the reaction was quenched with an equal volume of 250 mM ethylenediaminetetraacetic acid in 50% glycerol. The products were then analyzed using a 20% polyacrylamide/8 M urea/0.5X TBE gel with 0.5X TBE as the running buffer. The results were visualized with a Storm phosphorimager.

Fluorescence Anisotropy. Fluorescence emission intensities were collected on an Fluorolog-3 (SPEX) spectrofluorimeter equipped with a polarization assembly. The temperature was maintained with a thermostated compartment at 25 °C. HEX-labeled oligonucleotides were excited at 540 nm, and the resulting emission passed through a 550 nm cutoff filter (Oriel, Stratford, CT) or through a monochromator set at 556 nm. All samples were monitored with stirring using a nitric acid-cleaned quartz cuvette (NSG Scientific, Farmingdale, NY). At each concentration of enzyme, at least three readings were taken over a 2 s integration time and averaged. Anisotropy values were obtained in triplicate and automatically calculated from the equation

$$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) \quad (1)$$

where I is the recorded intensity at the indicated polarizer orientations and A is the anisotropy.

For competition fluorescence anisotropy experiments, increasing amounts (up to 15 equiv) of unlabeled duplex DNA were added to a preformed fluorescent DNA−*Pvu*II endonuclease complex and the resultant anisotropy was measured.

Determination of Equilibrium Constants. Equilibrium dissociation constants were determined in triplicate in 50 mM Tris and 100 mM NaCl at pH 7.5 and 25 °C except where otherwise indicated. The equilibrium constants weak enough to be measured spectroscopically were determined with a HEX-labeled 14-mer duplex using fluorescence anisotropy.

As appropriate, the corrected intensities or anisotropy values (A) were then plotted vs enzyme concentration and the data were fit to a simple binding isotherm (*19*) using Kaleidagraph 3.5 (Synergy, Reading, PA).

$$\theta = (K_a[E]_f)/(1 + K_a[E]_f) \quad (2)$$

where θ is the fraction of duplex bound, $[E]_f$ is the free enzyme concentration, and K_a is the association constant. The experiments were designed such that $[E]_f$ is approximated by $[E]_{total}$ (i.e., $5[DNA] \leq K_d$). The K_d values reported are averages of at least three measurements. The competition data were fit to a system of isotherms using Scientist software (MicroMath, Salt Lake City, UT) as previously described (*20*).

NMR Analysis. NMR samples contained 650 μM *Pvu*II monomers in 25 mM Tris, 200 mM KCl, and 10 mM $CaCl_2$ at pH* 7.7 and 25 °C. ^{1}H−^{15}N TROSY HSQC NMR spectra were collected on a 600 MHz Varian Inova spectrometer located at nearby Washington University. HSQC spectra were acquired with 128 transients of 1024 points in the ^{1}H dimension and 100 points in the ^{15}N dimension. The ^{1}H spectral width was 8385 Hz, and the ^{15}N spectral width was 1800 Hz. ^{1}H chemical shifts are relative to DSS.

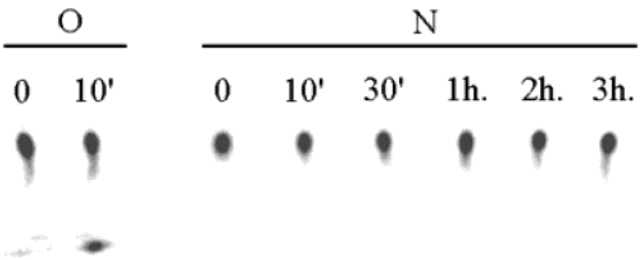

FIGURE 2: Denaturing PAGE analysis of *Pvu*II endonuclease cleavage reactions of SC14 containing native (O) and phosphoramidate (N) scissile linkages. Conditions: 125 nM duplex, 625 nM *Pvu*II endonuclease dimers in 50 mM Tris, 100 mM NaCl, and 10 mM $MgCl_2$ at pH 7.5 and 37 °C. The reaction times are as indicated.

^{1}H-decoupled ^{31}P NMR spectra were collected in 10% D_2O on a Bruker ARX 500 spectrometer at 202 MHz. Using a sweep width of 5100 Hz, FIDs of 700 points from 185 000 scans were acquired and processed with zero filling and line broadening (2.5 Hz). ^{31}P chemical shifts are relative to external trimethyl phosphate in D_2O.

The pH titration was conducted on a 0.65 mM N-SC14 sample in 1 mM Tris and 10% D_2O. ^{31}P NMR spectra were collected after the addition of HCl or NaOH as appropriate. The pH values were determined in a 5 mm NMR tube using an ultrathin NMR pH electrode (Wilmad, Buena, NJ). The measurements were made both before and after spectral acquisition and averaged.

RESULTS

The goal of this study was to characterize the interactions between the restriction enzyme *Pvu*II endonuclease and oligonucleotides featuring a phosphoramidate linkage at the scissile phosphate. To this end, a spectrum of kinetic, binding, and spectroscopic techniques was applied.

Cleavage Reactions. In the limited number of studies of phosphoramidate−nuclease interactions, it is generally observed that oligonucleotides containing this linkage are not cleaved (*4*, *10*). To determine if this is the case for *Pvu*II endonuclease, we conducted long reactions, incubating a 125 nM 5′-^{32}P-labeled N-SC14 duplex with an excess of enzyme (625 μm of enzyme dimers, single turnover conditions) at 10 mM $MgCl_2$. As shown in Figure 2, we were unable to detect cleavage of this substrate. This result extended to higher concentrations (500 nM duplex, 2.5 μM enzyme dimers) and longer reaction times (22 h; data not shown). Parallel results were obtained with N-NSC16 (data not shown).

To determine the effect of the presence of a phosphoramidate linkage on the cleavage of a native, complementary strand, we constructed two NSC16 heteroduplexes in which the top (N, O) and bottom (O, N) strands featured a phosphoramidate linkage at the scissile phosphate. As shown in Figure 3, cleavage of both native strands is observed in the heteroduplexes; no cleavage could be detected in analysis of duplexes in which the phosphoramidate strand was labeled. This observation extended to longer reaction times (up to 18 h; data not shown). In our experience, nonself-complementary duplexes are typically cleaved faster by *Pvu*II endonuclease than self-complementary duplexes (Conlan, L., and Bowen, L. Unpublished results).

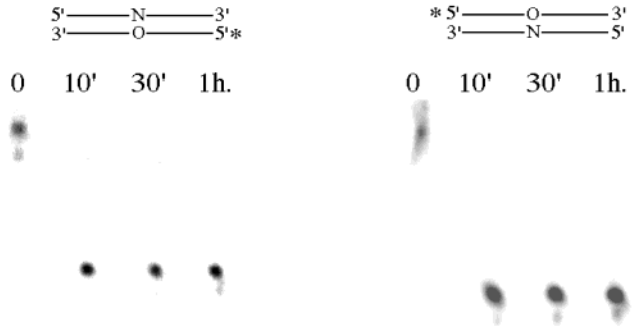

FIGURE 3: Denaturing PAGE analysis of *Pvu*II endonuclease cleavage reactions of NSC16 heteroduplex, which features a native (O) scissile phosphate on one strand and a phosphoramidate (N) scissile phosphate on the other strand. Conditions: 125 nM duplex, 625 nM *Pvu*II endonuclease dimers in 50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$ at pH 7.5 and 37 °C. The reaction times are as indicated. The difference in product mobility reflects the one nucleotide difference in length between the two strands. Typically, nonself-complementary duplexes are cleaved by *Pvu*II endonuclease faster than the self-complementary duplexes. See the text for details.

Metal Ion-Dependent DNA Binding. In our mechanistic studies, we have been especially interested in understanding how metal ions stimulate DNA binding. With native oligonucleotides, studies are limited to Ca(II), which does not support turnover. A nonhydrolyzable analogue provides a rare opportunity to examine differential abilities of Ca(II) and Mg(II) as DNA binding cofactors.

Because of our success with nitrocellulose filter binding (*13*, *21*) and a previous report of success with this method with other systems (*10*), we began with this technique. Despite the ease with which binding constants were obtained for native, control oligonucleotides, we were unable to obtain reliable data with phosphoramidate−*Pvu*II samples. Experimenting with filters from different sources, with differing pore sizes, and large arrays of experimental conditions did not produce a discernible binding trend. In all cases, remarkable retention of the phosphoramidate on the filter was observed.

We therefore turned to fluorescence anisotropy, a technique that has been successfully applied to *Pvu*II endonuclease and other restriction enzymes (*13*, *20*). By working with oligonucleotides end-labeled with HEX, a wide range of binding constants (1 nM to low micromolar) can be directly obtained with no appreciable dye interference (*13*, *20*). Dissociation constants tighter than 1 nM can either be estimated or be quantitated via competition experiments (*13*). To this end, a less expensive, self-complementary, HEX-tagged 14-mer duplex was designed (HEX−N-SC14). Because of the reverse direction of synthesis for this phosphoramidate, the HEX tag was placed on the 3′-end of the duplex. It is attached to the oligonucleotide via a carboxyhexyl linker (www.glenres.com).

Direct fluorescence titrations of the enzyme into the HEX−N-SC14 duplex in the presence of 10 mM $CaCl_2$ quickly revealed subnanomolar binding affinity. Using the results of filter binding experiments with O-SC14 (Table 2), we applied competition fluorescence anisotropy to quantitate *Pvu*II−phosphoramidate binding affinity in the presence of Ca(II) (Figure 4). In this experiment, O-SC14 was titrated into a HEX−N-SC14−enzyme complex and the K_d for HEX−N-SC14 was determined from simultaneous fits of both equilibria. As summarized in Table 2, native and phosphoramidate duplexes bound the enzyme with similar affinities. Both K_d values were near 200 pM and quite comparable to values reported for other cognate duplexes binding *Pvu*II endonuclease (*13*, *22*).

Table 2: Summary of Native and Phosphoramidate DNA Binding by *Pvu*II Endonuclease[a]

oligonucleotide	method	metal ions	dye	K_d
native (O-SC14)	anisotropy	metal-free	HEX	232 ± 30 nM
	filter binding	Ca(II)	none	398 ± 190 pM
phosphoramidate (N-SC14)	anisotropy	metal-free	HEX	5.4 ± 4.1 nM
	anisotropy	Ca(II)	none/HEX	239 ± 145 pM[b]
	anisotropy	Mn(II)	HEX	(500 pM)[c]
	anisotropy	Mg(II)	HEX	12 ± 4 nM

[a] Experiments were performed with native (O) and phosphoramidate (N) SC14 and HEX−SC14 as described in the text. Conditions: 50 mM Tris and 100 mM NaCl at pH 7.5 and 25 °C unless otherwise indicated. In experiments where metal ions were present, the concentration of the metal ion chloride salt was 10 mM, and the NaCl concentration was adjusted to 70 mM to maintain constant ionic strength relative to metal-free conditions. All values reported in the table are averages of at least three measurements. [b] Obtained in competition with native oligonucleotide duplex. See Figure 4. [c] Because of fluorescence detection limits, the duplex concentration was similar to the K_d.

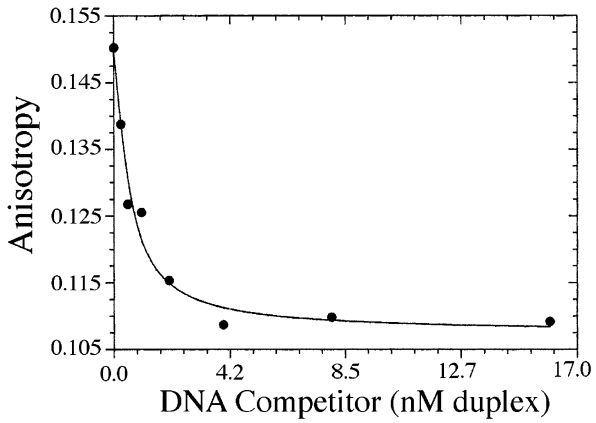

FIGURE 4: Ca(II)-dependent phosphoramidate DNA binding by *Pvu*II endonuclease as measured by competition fluorescence anisotropy. The unlabeled O-SC14 duplex was titrated into 0.5 nM preformed phosphoramidate HEX−N-SC14−enzyme complex. Conditions: 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$ at pH 7.5 and 25 °C. The calculated K_d is 239 ± 145 pM for the phosphoramidate DNA duplex.

The stability of the phosphoramidate oligonucleotide toward *Pvu*II endonuclease activity permits the measurement of binding constants in the presence of Mg(II) and Mn(II), both of which promote cleavage of the native duplex. As summarized in Table 2, K_d values obtained in the presence of Mn(II) are comparable to those obtained in the presence of Ca(II). Figure 5A illustrates that Mg(II)-supported binding is somewhat weaker than that observed with Ca(II) and Mn(II), i.e., $K_d \approx 10$ nM.

Metal Ion-Independent DNA Binding. Quantitation of DNA binding in the absence of metal ions is critical to our understanding of the role of divalent metal ions in this process. To that end, metal-free DNA binding constants were measured for both native (O) and phosphoramidate (N) SC14 complexes. The binding constant for the native duplex under metal-free conditions is approximately 240 nM (Table 2). This value is nearly identical to what we observed with other *Pvu*II cognate oligonucleotides under metal-free conditions

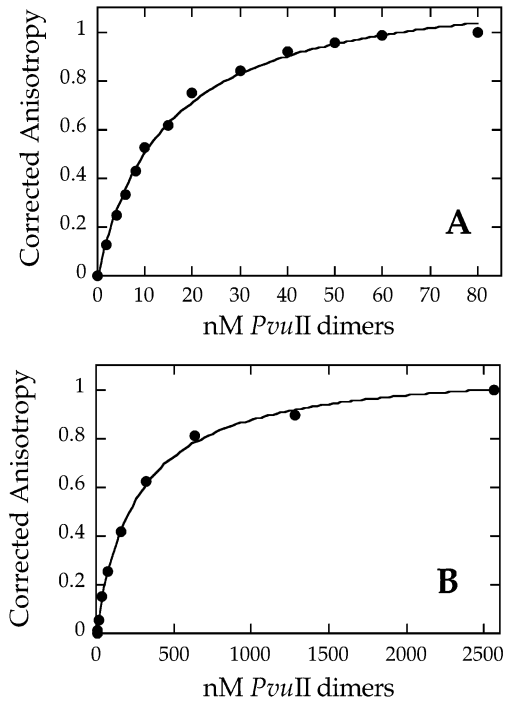

FIGURE 5: Phosphoramidate DNA binding isotherms. (A) In the presence of 10 mM $MgCl_2$. The concentration of HEX−N-SC14 was 2 nM. The initial and final anisotropy values were 0.0875 and 0.1560, respectively. The K_d value averaged from three curves is 12 ± 4 nM. (B) Under metal free conditions. The HEX−N-SC14 duplex concentration was 1 nM. The initial and final anisotropy values were 0.0808 and 0.1778, respectively. $K_d = 5.4 \pm 4.1$ nM. Conditions: 50 mM Tris, 100 mM NaCl at pH 7.5 and 25 °C, except where otherwise noted.

(*13*, *21*). However, as shown in Figure 5B and summarized in Table 2, metal-free binding of the enzyme to N-NSC14 is much tighter, exhibiting dissociation constants around 5 nM. This corresponds to a 50-fold greater affinity of the enzyme for the phosphoramidate oligonucleotide relative to the corresponding native duplex.

Conformational Analysis of Enzyme−DNA Complexes. One possible explanation for the inactivity of the enzyme toward phosphoramidate linkage is that the modification causes changes in oligonucleotide conformation, which may be manifested not only in the oligonucleotide but also in the enzyme−substrate complex. Indeed, both NMR and X-ray crystallographic studies of phosphoramidates indicate predominantly A-form sugar puckering (*7*, *8*), a conformational feature that is more typical of RNA than it is of B-form DNA.

One advantage of working with *Pvu*II endonuclease is that we have already established NMR spectroscopy as a solution probe of conformation (*14*, *15*). Simple survey experiments provide a level of detail that is not available by other spectroscopic means. To that end, we began by collecting ^{31}P NMR spectra of both the native and the phosphoramidate oligonucleotides. As shown in Figure 6, both oligonucleotides feature the typical envelope of resonances at −4 to −5 ppm. A few features distinguish the phosphoramidate spectrum. First, there is a distinct signal at 3.3 ppm, which can be attributed to the phosphoramidate linkage (*3*, *7*). On the basis of exchange behavior observed in pH and variable temperature experiments, we attribute the smaller peak slightly more upfield to a minor conformer of this center (*9*, *23*). Next, the remaining envelope of resonances is noticeably more dispersed; that is, the phosphates are more distinct from one another than in the native spectrum. This is evident on both sides of the envelope. Thus, it appears that the effect of the substitution on the phosphate backbone extends beyond the linkage itself, decreasing the homogeneity of at least two of the remaining phosphodiester linkages relative to the rest of the phosphates.

Given the discernible differences in enzyme affinities for native and modified substrates, we were especially interested in any differential effects of enzyme binding on phosphate backbone conformation and vice versa. Because of conditions necessary for NMR spectroscopy, native and phosphoramidate complexes with the enzyme are most easily compared in the presence of Ca(II). To conserve the costly phosphoramidate, one sample was prepared for both enzyme and DNA conformational analyses. Because excess enzyme would seriously compromise the quality and interpretability of HSQC spectra, an excess of DNA was mixed with the enzyme. This composition does not compromise the quality and interpretability of the ^{31}P NMR spectra.

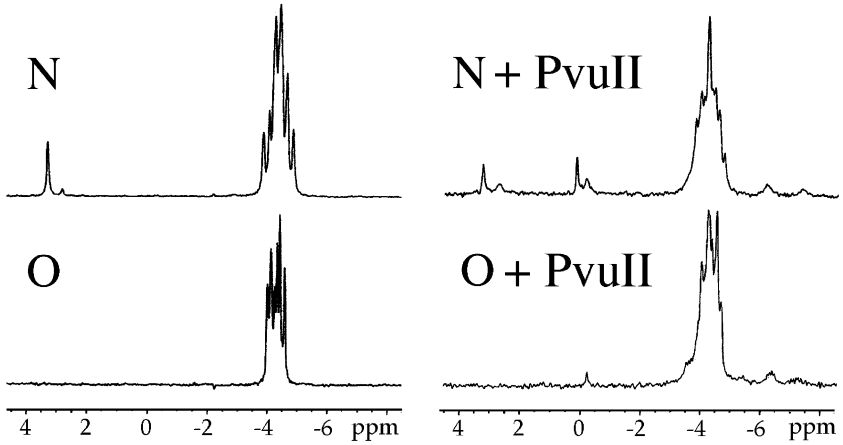

FIGURE 6: ^{1}H-decoupled ^{31}P NMR spectra of native (O) and phosphoramidate (N) SC14 duplexes in the absence and presence of wildtype *Pvu*II endonuclease. Conditions: 0.65 mM enzyme monomers, excess SC14 strands, 25 mM Tris, 200 mM KCl, 10 mM $CaCl_2$, 10% D_2O at pH* 7.7 and 25 °C. FIDs of 700 points from 185 000 scans were processed with zero filling and line broadening (2.5 Hz). Chemical shifts are relative to external trimethyl phosphate. Sharp intensities near 0 ppm are attributed to residual phosphate buffer from enzyme purification. See the text for a detailed interpretation.

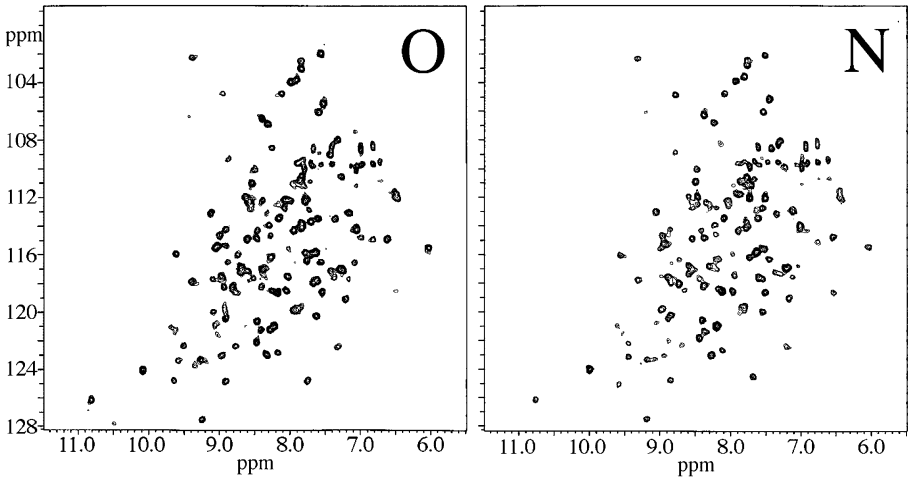

FIGURE 7: $^{1}H-^{15}N$ TROSY HSQC spectra of *Pvu*II endonuclease in the presence of native (O) and phosphoramidate-containing (N) SC14 duplexes at 600 MHz. Conditions: 0.65 mM enzyme monomers, excess SC14 strands, 25 mM Tris, 200 mM KCl, 10 mM $CaCl_2$, 10% D_2O at pH* 7.7 and 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

The ^{31}P NMR spectra of these complexes are featured in Figure 6. The spectra of native and phosphoramidate−enzyme complexes have a number of common features. Spectral broadening indicative of enzyme binding is evident in the peak envelope around −4 to −5 ppm. The sharper intensity near 0 ppm is commonly observed in enzyme samples and is attributed to residual phosphate buffer from protein purification. Also common to both spectra are a number of dramatically upfield-shifted ^{31}P resonances between −5 and −8 ppm. While crystallographic studies do not reveal significant distortions in the DNA when it is complexed to *Pvu*II endonuclease (*24*, *25*), we nevertheless attribute these intensities to backbone phosphodiester linkages that become conformationally distinct from the envelope upon enzyme binding (*26*). Although we cannot rule out some subtle differences, the fact that such patterns are present in both samples indicates a similarity in the binding interactions with the enzyme.

There are also a number of features that are unique to the phosphoramidate−enzyme complex. The peak at 3.3 ppm is still present but is smaller, indicative of enzyme binding in slow exchange. The increased line width of the smaller, more upfield peak attributed to a minor conformer is also consistent with enzyme binding and/or exchange broadening. This leaves the small broad peak just upfield from residual phosphate (≈ -0.25 ppm). The line width of this resonance is also consistent with enzyme binding. In separate experiments where additional enzyme is added, this peak increases in intensity while that of the peak at 3.3 ppm is correspondingly smaller (data not shown). While we cannot rule out a downfield shift for a resonance from the large envelope, we tentatively assign this peak to the enzyme-bound phosphoramidate linkage. If this is the case, the dramatic difference in chemical shift relative to the free phosphoramidate resonance is consistent with some alteration in the conformation of the phosphoramidate linkage upon enzyme binding.

Conversely, enzyme conformation in the presence of native and phosphoramidate oligonucleotides was evaluated using two-dimensional $^{1}H-^{15}N$ HSQC NMR spectroscopy. In these experiments, amide protons are correlated to their corresponding nitrogens, providing a "fingerprint" spectrum of the enzyme (*27*). Figure 7 features the HSQC spectra of *Pvu*II endonuclease complexed to O-SC14 and N-SC14. Ideally, approximately 170 cross-peaks are expected resulting from backbone and side chain amide groups of the enzyme. However, because *Pvu*II endonuclease is a large protein by NMR standards (2 × 18 kDa), it is reasonable that about 60% of the resonances are visible under these experimental conditions; the remainder likely corresponds to spectral overlap and resonances with poor signal-to-noise ratios due to unfavorable exchange and/or relaxation behavior. Even with this fraction of visible peaks, such HSQC spectra provide valuable information about how ligands affect backbone conformational behavior.

As illustrated, the backbone conformations of *Pvu*II endonuclease when bound to these oligonucleotides are quite similar. This is particularly evident between 10 and 11 ppm, where the indole nitrogen protons typically resonate, and between 7.50 and 8.55 at about 104 ppm ^{15}N. There are a number of small differences in the spectra, but the overall impression is one of general similarity between the two complexes. Taken altogether, these spectral results indicate that while there may be local differences, there are no extensive conformational differences between native and phosphoramidate enzyme−DNA complexes.

DISCUSSION

Backbone-Modified Analogues of Nucleic Acids. In an effort to understand the behavior of nucleic acids and their interaction with protein ligands, a number of research groups have pursued studies of nucleic acids with modified backbones. Of particular interest here are those featuring modified phosphodiester linkages.

The most classic of these are the phosphorothioates, which feature sulfur substitution at nonbridging oxygens of phosphodiester linkages. These chiral analogues were used to establish the inversion of configuration at phosphorus that is characteristic of restriction enzyme reactions (*28*). Oligonucleotides featuring substitution with sulfur at the 3′-bridging oxygen of the scissile phosphate, termed phosphorothiolates, are also resistant to cleavage by restriction

enzymes. In gel mobility shift experiments, *Eco*RV endonuclease was found to bind modified oligonucleotides in the absence of metal ions, but affinity was not enhanced by the presence of $MgCl_2$ (*29*). The structural basis for this behavior is elaborated in a later crystallographic study in which this behavior was attributed to conformational distortions proximal to the sulfur atom (*30*).

Considerably less is known about the interactions of enzymes with phosphoramidates, in which the 3′-bridging oxygen is replaced by nitrogen rather than sulfur. Gromova et al. described synthetic strategies and conducted a pilot study of phosphoramidate duplex interactions with the restriction enzymes *Eco*RII and *Sso*II. The phosphoramidate linkage was found to be resistant to cleavage in both cases. The former enzyme was found to cleave opposing native strands in heteroduplexes (*4*, *10*). They noted that the K_d for phosphoramidate binding (presumably in the absence of added metal ions) was 11 nM; in the presence of 5 mM $MgCl_2$, the K_d is 30 nM.

Here, we add considerable dimension to our understanding of phosphoramidate−restriction enzyme interactions. The studies described here provide two critical advantages: One, our studies are conducted in a well-defined system, with a host of functioning equilibrium and spectroscopic assays (*13*, *15*). Two, we quantitatively compare binding affinities with those observed in the presence of Ca(II) and, most importantly, with native oligonucleotides in the same oligonucleotide sequence. This allows for the most complete analysis of metalloendonuclease−phosphoramidate interactions reported to date.

DNA Binding in the Presence of Metal Ions. As with native oligonucleotides, Ca(II) significantly stimulates phosphoramidate binding by the enzyme. Binding constants are about 20-fold stronger in the presence of $CaCl_2$ than under metal-free conditions. This factor is more modest than the 600-fold difference that we observe with the corresponding native oligonucleotide used in these studies or the slightly larger factors that we have obtained for other native cognate oligonucleotides (*13*). A similar pattern is exhibited by other restriction enzyme systems, for which Ca(II)-stimulated DNA binding is well over 100-fold better than metal ion-independent DNA binding (*13*). In this respect, phosphoramidate duplexes behave very much like native duplexes.

In the presence of Mg(II), the determined binding constant for the phosphoramidate duplex is comparable to that of the metal-free value, representing no measurable stimulation of DNA binding by this cofactor. Because of the especially avid metal-free binding of the phosphoramidate, we were concerned that such a pattern might be specific to the phosphoramidate substrate. We attempted to address this issue with the *Pvu*II active site variant K70A. This variant binds cognate DNA with strength and specificity and exhibits only residual activity in λ-cleavage assays (*22*). However, in single turnover cleavage assays under conditions needed for our binding measurements, this activity was significant enough to make binding measurements with Mg(II) uninterpretable.

This leaves literature examples to provide perspective. With phosphorothiolate duplexes, no enhancement of cognate DNA binding by *Eco*RV endonuclease was observed in the presence of Mg(II) relative to metal-free conditions (*29*). A similar measurement with a nonhydrolyzable thioribose substrate analogue revealed only a 4-fold enhancement in affinity in the presence of Mg(II) (*31*). In contrast, a binding enhancement of 80 ($K_a^{Mg(II)}/K_a^{metal-free}$) was observed with a nonhydrolyzable DNA in which the central T of the *Eco*RV recognition sequence 5′-GATATC-3′ was replaced with U (*32*). Upon considering the data available, it appears that while specific enhancements vary somewhat, Mg(II) appears to stimulate DNA binding by restriction enzymes. We speculate that since metal-free binding of native duplexes is so weak and we can easily observe cleavage at low nanomolar duplex concentrations (data not shown), Mg(II) most likely stimulates native DNA binding by *Pvu*II endonuclease.

Ca(II) vs Mg(II). One of the motivations of this study was to compare Mg(II) and Ca(II) as stimulants of DNA binding by restriction enzymes. Ca(II) is used as a mimic for Mg(II) for two reasons: (i) It stimulates DNA binding but does not support cleavage, and (ii) metal ion substitution is significantly more convenient than the preparation of nonhydrolyzable DNA analogues. Ca(II) and Mg(II) have a number of similarities, including shared ligand and geometry preferences (*33*). However, Ca(II) is considerably larger than Mg(II) (0.99 vs 0.65 Å), which we have reasoned to be the source of Ca(II)-dependent conformational changes in *Pvu*II endonuclease (*14*).

In one *Eco*RV study with nonhydrolyzable thioribose-substituted DNA, Ca(II) stimulated DNA binding about 200-fold more effectively than Mg(II) (*31*). In a subsequent study of the same system involving oligonucleotide duplexes featuring the U substitution, Ca(II) stimulated DNA binding about 4-fold better than Mg(II). In the same study, Ca(II)-supported DNA binding constants for the relatively inactive K38A *Eco*RV endonuclease were about 50-fold better than those measured in the presence of Mg(II) (*32*). While the particulars vary somewhat, the basic observation for *Eco*RV endonuclease is that Ca(II) stimulates DNA binding more efficiently than does Mg(II). The data presented here for *Pvu*II endonuclease appear to be consistent with this pattern: In this system, Ca(II) is about 50-fold better than Mg(II) at stimulating binding at the same metal ion concentration.

Of course, the remaining question is why differences among Ca(II), Mn(II), and Mg(II) are observed. To address this, the extent to which this is a function of the phosphoramidate must first be considered. One possibility is that the phosphoramidate linkage interacts with the metals differently. This is most easily justified for Mn(II), which displays a greater preference for nitrogen ligands than Ca(II) or Mg(II) (*33*). However, Ca(II) and Mg(II) differ very little in ligand preference; both are hard metal ions that prefer oxygen ligands and thus would not be likely to be differentially affected by the substitution to nitrogen at the scissile phosphate. On the other hand, as discussed by Martin et al. (*32*), differences in ionic radius between these two ions and flexible coordination numbers for Ca(II) could easily lead to local conformational differences that favor Ca(II). In other words, the flexible coordination behavior of Ca(II) permits the assembly of a wider variety of geometries than Mg(II), any number of which are consistent with successful complex formation. Mg(II) ligand geometries are more constrained to six ligand, octahedral systems, putting it at a disadvantage relative to Ca(II) in this regard.

While this does address why Ca(II) is able to promote DNA binding under conditions in which Mg(II) cannot, it does not necessarily explain why DNA binding in the

presence of Ca(II) is tighter than it is with Mg(II). We have previously observed by ^{19}F NMR spectroscopy that Ca(II) induces fairly substantial conformational changes in *Pvu*II endonuclease (*14*). It is possible that these conformational changes are leveraged into a better fit with DNA, forming a tighter complex. This avenue is not accessible to Mg(II), which does not promote conformational changes in the free enzyme (*14*).

DNA Binding in the Absence of Metal Ions. The most remarkable observation of this study is how tightly the enzyme binds phosphoramidate duplexes in the absence of metal ions. Our measured binding constant of 5 nM under these conditions is consistent with the enzyme−phosphoramidate K_d reported for *Eco*RII (*10*) and interestingly quite similar to metal-free DNA binding constants obtained by Reid et al. for *Eco*RV endonuclease (*20*). However, because we also measured the K_d for a native oligonucleotide of the same sequence in the absence of metal ions, we can put this result into better perspective. *Pvu*II endonuclease binds the phosphoramidate oligonucleotide about 40-fold more tightly (2.2 kcal/mol) than the corresponding native duplex.

One simplistic rationalization for this behavior is that in the absence of divalent cations, there are electrostatic differences that might reduce repulsions between polyanionic DNA and acidic active site residues (Glu68, Asp58, and Glu55). However, while nitrogen is less electronegative than oxygen, by all accounts the phosphoramidate center remains negatively charged (*6*). Indeed, pH titrations of the phosphoramidate linkage as monitored by ^{31}P NMR spectroscopy indicate that there are no transitions in the physiological pH range, a behavior that it shares with native phosphodiester linkages (see the Supporting Information). Thus, it follows that while there are likely to be some subtle differences in charge distribution at that scissile linkage, the phosphoramidate linkage is generally as electrostatically repulsive to active site groups as the native linkage.

Less obvious is why native and phosphoramidate binding constants are so similar in the presence of Ca(II) when metal-free K_d values are so different. Clearly, factors that contribute to avid substrate binding in the presence of Ca(II) dominate any effects from differences in DNA structure. Perhaps without the Ca(II)-driven enzyme conformational changes that accompany the assembly of the ternary complex, the enzyme is more sensitive to structural differences in the scissile linkage. Analogous to the effect of Ca(II) on enzyme conformation, perhaps the perturbation in sugar puckering is somehow energetically leveraged into the formation of a tighter metal-free complex.

Phosphoramidate Stability toward Nucleases. We are interested not only in the nuclease binding behavior of phosphoramidates but their resistance to cleavage as well. Previous studies of nuclease resistance of phosphoramidates involved primarily DNA and RNA oligonucleotides composed entirely of these linkages. Such nucleic acids were found to be stable in the presence of nonspecific nucleases such as snake venom phosphodiesterase and those found in cellular extracts (*5*).

There are a number of factors that could potentially affect nuclease resistance. One is duplex stability. However, the T_m values for uniform phosphoramidate oligonucleotides Watson−Crick duplexes are greater than those of native duplexes (*2*). Our data indicate that phosphoramidate duplexes bind *Pvu*II endonuclease more avidly than native oligonucleotides, so resistance to cleavage cannot be attributed to poor affinity. Interestingly, the inherent reactivity of a phosphoramidate linkage is not an issue, either. A recent study of diribonucleotide phosphoramidates indicates that under alkaline conditions, phosphoramidates are hydrolyzed twice as quickly as the corresponding native linkage. Under acidic conditions, the difference is more dramatic: phosphoramidates are 300-fold less stable than native ribonucleotides (*34*). Clearly, other factors are responsible for the stability of phosphoramidates against nucleases.

Conformational Effects. Our NMR data clearly indicate that in the presence of Ca(II), the complexes that the enzyme forms with native and phosphoramidate duplexes are quite similar. However, it is entirely plausible that subtle differences exist at the enzyme−DNA interface to which both enhanced affinity and resistance to cleavage could be attributed. One unique feature of phosphoramidates is that the decreased electronegativity of the N atom relative to O forces a C3′ endo sugar pucker (*6*, *7*, *9*). Our observation by ^{31}P NMR spectroscopy of decreased degeneracy in backbone phosphate conformations supports the existence of local perturbations, which could extend to duplexes bound to the enzyme. Such perturbations could move critical reacting groups out of proper alignment for catalysis. Literature studies lend support to this proposal. Indeed, substitution with sulfur in the ribose ring (thioribose) does promote differences in sugar puckering in a free DNA duplex (*35*). When complexed with *Eco*RV endonuclease, however, this is not as obvious (*29*). In contrast, phosphorothiolate substitution at the scissile linkage promotes significant local perturbations in the *Eco*RV endonuclease−DNA complex (*30*). This could easily be occurring in *Pvu*II−phosphoramidate complexes.

While the argument that conformation is important might initially seem dismissive, it is worth noting that restriction enzymes have an elegant sensitivity to such structural subtleties. In more than one restriction enzyme, mutation of amino acids involved in base contacts results not only in a relaxation of sequence specificity but a loss of catalytic activity as well (*22*, *36*, *37*). Such sensitivities of course extend to the catalytic metal ion binding site. As already mentioned, Ca(II) promotes avid DNA binding but not cleavage by restriction enzymes (*13*). We elaborated on this sensitivity in a recent study of *Pvu*II endonuclease, in which we found that other metal ion substitutions can have even more dramatic effects on DNA binding affinity, sequence specificity, and cleavage activity (*21*). Therefore, what can be rationalized as subtle differences in metal ion properties are propagated into devastating effects on the multiple functions of this enzyme.

CONCLUSION

We have examined the reactivity and affinity of *Pvu*II endonuclease for oligonucleotides featuring scissile phosphoramidate linkages. Quantitative substrate binding studies indicate that for this system, Ca(II) is more effective than Mg(II) at promoting cognate DNA binding.

NMR spectroscopic analysis indicates that there are not likely to be any global conformational differences between native and phosphoramidate enzyme−DNA complexes in the

presence of Ca(II). However, when all of the evidence is considered, it appears that the O→N substitution probably results in differences in local conformation that are in turn responsible for a lack of reactivity and increased affinity. This perturbation could be more obvious in metal-free complexes. Characterizing the precise nature of these features would be a worthy crystallographic study. In the meantime, phosphoramidate duplexes should prove useful in the study of stable restriction enzyme−substrate complexes in the presence of Mg(II).

SUPPORTING INFORMATION AVAILABLE

^{31}P NMR spectra of the 14-mer phosphoramidate duplex as a function of pH. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Kurreck, J. (2003) Antisense technologies, *Eur. J. Biochem. 270*, 1628−1644.
2. Gryaznov, S., Lloyd, D. H., Chen, J.-K., Schultz, R. G., DeDionsio, L. A., Ratmeyer, L., and Wilson, W. D. (1995) Oligonucleotide N3′→P5′ phosphoramidates, *Proc. Natl. Acad. Sci. U.S.A. 92*, 5798−5802.
3. Gryaznov, S., and Winter, H. (1998) RNA mimetics: oligoribonucleotides N3′→P5′ phosphoramidates, *Nucleic Acids Res. 26*, 4160−4167.
4. Yolov, A., Gromova, E., Kubareva, E., Potapov, V., and Shabarova, Z. (1985) Interaction of *Eco*RII restriction and modification enzymes with synthetic DNA fragments. V. Study of single-strand cleavages, *Nucleic Acids Res. 13*, 8969−8981.
5. Gryaznov, S., Skorski, T., Cucco, C., Nieborowska-Skorska, M., Chiu, C. Y., Lloyd, D., Chen, J.-K., Koziolkiewicz, M., and Calabretta, B. (1996) Oligonucleotide N3′→P5′ phosphoramidates as antisense agents, *Nucleic Acids Res. 24*, 1508−1514.
6. Ding, D., Gryaznov, S., Lloyd, D., Chandrasekaran, S., Yao, S., Ratmeyer, L., Pan, Y., and Wilson, W. (1996) An oligodeoxyribonucleotide N3′→P5′ phosphoramidate duplex forms an A-type helix in solution, *Nucleic Acids Res. 24*, 354−360.
7. Ding, D., Gryaznov, S., and Wilson, W. (1998) NMR solution structure of the N3′→P5′ phosphoramidate duplex d(CGCGAATTCGCG)$_2$ by the iterative relaxation matrix approach, *Biochemistry 37*, 12082−12093.
8. Tereshko, V., Gryaznov, S., and Egli, M. (1998) Consequences of replacing the DNA 3′-oxygen by an amino group: High-resolution crystal structure of a fully modified N3′→P5′ phosphoramidate DNA dodecamer duplex, *J. Am. Chem. Soc. 120*, 269−283.
9. Banavali, N., and MacKerll, A., Jr. (2001) Reexamination of the intrinsic, dynamic and hydration properties of phosphoramidate DNA, *Nucleic Acids Res. 29*, 3219−3230.
10. Gromova, E., Vinogradova, M., Uporova, T., Griaznova, O., Isaguliants, M., Kosykh, V., Nikolskaya, I., and Shabarova, Z. (1987) DNA-duplexes with phosphoamide bonds: interaction with restriction endonucleases *Eco*RII and *Sso*II, *Bioorg. Khim. 13*, 269−272.
11. Pingoud, A., and Jeltsch, A. (1997) Recognition and cleavage of DNA by type-II restriction endonucleases, *Eur. J. Biochem. 246*, 1−22.
12. Pingoud, A., and Jeltsch, A. (2001) Structure and function of type II restriction endonucleases, *Nucleic Acids Res. 29*, 3705−3727.
13. Conlan, L. H., and Dupureur, C. M. (2002) Dissecting the metal ion dependence of DNA binding by *Pvu*II endonuclease, *Biochemistry 41*, 1335−1342.
14. Dupureur, C. M., and Hallman, L. M. (1999) Effects of divalent metal ions on the activity and conformation of native and 3-fluorotyrosine-*Pvu*II endonucleases, *Eur. J. Biochem. 261*, 261−268.
15. Dupureur, C. M., and Dominguez, J., M. A. (2001) The PD··· (D/E)XK motif in restriction enzymes: A link between structure and conformation, *Biochemistry 40*, 387−394.
16. Dominguez, M. A., Jr., Thornton, K. C., Melendez, M. G., and Dupureur, C. M. (2001) Differential effects of isomeric incorporation of fluoro-phenylalanines into *Pvu*II endonuclease, *Proteins 45*, 55−61.
17. Holmquist, B. (1988) Elimination of adventitious metals, *Methods Enzymol. 158*, 6−12.
18. Wagner, F. W. (1988) Preparation of metal-free enzymes, *Methods Enzymol. 158*, 21−32.
19. Heyduk, T., and Lee, J. C. (1990) Application of fluorescence energy transfer and polarization to monitor *Escherichia coli* cAMP receptor protein and lac promoter interaction., *Proc. Natl. Acad. Sci. U.S.A. 87*, 1744−1748.
20. Reid, S. L., Parry, D., Liu, H.-H., and Connolly, B. A. (2001) Binding and recognition of GATATC target sequences by the *Eco*RV restriction endonuclease: a study using fluorescent oligonucleotides and fluorescence polarisation, *Biochemistry 40*, 2484−2494.
21. Bowen, L. M., and Dupureur, C. M. (2003) Investigation of restriction enzyme cofactor requirements: A relationship between metal ion properties and sequence specificity, *Biochemistry 42*, 12643−12653.
22. Nastri, H. G., Evans, P. D., Walker, I. H., and Riggs, P. D. (1997) Catalytic and DNA binding properties of *Pvu*II restriction endonuclease mutants, *J. Biol. Chem. 272*, 25761−25767.
23. Banavali, N., and MacKerll, A., Jr. (2001) Reevaluation of stereoelectronic contributions to the conformational properties of the phosphodiester and N3′-phosphoramidate moieties of nucleic acids, *Nucleic Acids Res. 29*, 3219−3230.
24. Cheng, X., Balendiran, K., Schildkraut, I., and Anderson, J. E. (1994) Structure of *Pvu*II endonuclease with cognate DNA, *EMBO J. 13*, 3927−3935.
25. Horton, J. R., and Cheng, X. (2000) *Pvu*II endonuclease contains two calcium ions in active sites, *J. Mol. Biol. 300*, 1049−1056.
26. Gorenstein, D. G. (1994) Conformation and dynamics of DNA and protein-DNA complexes by ^{31}P NMR, *Chem. Rev. 94*, 1315−1338.
27. Roberts, G. C. K. (1993) *NMR of Macromolecules*, IRL, New York.
28. Connolly, B. A., Eckstein, F., and Pingoud, A. (1984) The stereochemical course of the restriction endonuclease *Eco*RI-catalyzed reaction, *J. Biol. Chem. 259*, 10760−10763.
29. Szczelkun, M. D., and Connolly, B. A. (1995) Sequence-specific binding of DNA by the *Eco*RV restriction and modificiation enzymes with nucleic acid and cofactor analogues, *Biochemistry 34*, 10724−10733.
30. Horton, N. C., Connolly, B. A., and Perona, J. J. (2000) Inhibition of *Eco*RV endonuclease by deoxyribo-3′-S-phosphorothioates: A high-resolution X-ray crystallographic study, *J. Am. Chem. Soc. 122*, 3314−3324.
31. Engler, L. E., Welch, K. K., and Jen-Jacobson, L. (1997) Specific binding by *Eco*RV endonuclease to its DNA recognition site GATATC, *J. Mol. Biol. 269*, 82−101.
32. Martin, A. M., Horton, N. C., Lusetti, S., Reich, N. O., and Perona, J. J. (1999) Divalent metal dependence of site-specific DNA binding by *Eco*RV endonuclease, *Biochemistry 38*, 8430−8439.
33. Cowan, J. A. (1995) *The Biological Chemistry of Magnesium*, VCH, NY.
34. Ora, M., Mattila, K., Lonnberg, T., Oivanen, M., and Lonnberg, H. (2002) Hydrolytic reactions of diribonucleoside 3′5′-(3′-N-phosphoramidates): Kinetics and mechanisms for the P−O and P−N bond cleavage of 3′-amino-3′-deoxyuridylyl-3′,5′-uridine, *J. Am. Chem. Soc. 124*, 14364−14372.
35. Boggon, T. J., Hancox, E. L., McAuley-Hecht, K. E., Connolly, B. A., Hunter, W. N., Brown, T., Walked, R. T., and Leonard, G. A. (1996) The crystal structure analysis of d(CGCGAASSCGCG)$_2$, a synthetic DNA dodecamer duplex containing four 4′-thio-2′-deoxythymidine nucleotides, *Nucleic Acids Res. 24*, 951−961.
36. Horton, J. R., Nastri, H. G., Riggs, P. D., and Cheng, X. (1998) Asp34 of *Pvu*II endonuclease is directly involved in DNA minor groove recognition and indirectly involved in catalysis, *J. Mol. Biol. 284*, 1491−1504.
37. Jeltsch, A., Alves, J., Oelgeschlager, T., Wolfes, H., Maass, G., and Pingoud, A. (1993) Mutational analysis of the function of Gln115 in the *Eco*RI restriction endonuclease, a critical amino acid for recognition of the inner thymidine residue in the sequence -GAATTC- and for coupling specific DNA binding to catalysis, *J. Mol. Biol. 229*, 221−234.

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