Dissecting the Metal Ion Dependence of DNA Binding by *Pvu*II Endonuclease†

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ABSTRACT: Divalent cations can provide an effective means of modulating the behavior of nucleic acid binding proteins. As a result, there is strong interest in understanding the role of metal ions in the function of both nucleic acid binding proteins and their enzymes. We have applied complementary fluorescence spectroscopic and nitrocellulose filter binding assays to quantitate the role of metal ions in mediating DNA binding and sequence specificity by the representative *Pvu*II endonuclease. At pH 7.5 in the presence of the catalytically nonsupportive Ca(II), this enzyme binds the *Pvu*II target sequence with a K_d of 50 pM. Under strict metal-free conditions, the enzyme exhibits a K_d of only 300 nM for the cognate sequence, an affinity which is weak relative to those measured for other systems in the absence of metal ions. This represents a 6000-fold increase in *Pvu*II affinity for cognate DNA upon the addition of Ca(II). The pH dependences of both metal ion-dependent and metal ion-independent DNA binding are remarkably shallow throughout the physiological range; other characterized restriction enzymes exhibit more pronounced pH dependences of DNA binding even in the absence of metal ions. Similar measurements with noncognate sequences indicate that divalent metal ions are not important to nonspecific DNA binding; K_d values are ≈200 nM throughout the physiological pH range, a behavior shared with other endonucleases. While some of these results extend somewhat the range of expected behavior for restriction enzymes, these results indicate that *Pvu*II endonuclease shares with other characterized systems a mechanism by which cognate affinity and sequence discrimination are most effectively achieved in the presence of divalent metal ions.

Enzymes that hydrolyze nucleic acids evolve to neutralize the strongly anionic charge of the substrate and subsequently mediate catalysis. Divalent metal ions are uniquely suited for participation in these mechanistic duties. Perhaps due the universal nature of nuclease activity, many groups are interested in understanding exactly how divalent metal ions support this common hydrolytic activity. Among the best studied nucleases are type II restriction endonucleases, Mg(II)-dependent enzymes which recognize and cleave 4−6 bp sequences of DNA with a high degree of specificity (*1*).

A number of laboratories have been interested in quantitating the influence of divalent metal ions on DNA affinity and sequence specificity of restriction enzymes. In the absence of divalent metal ions, the classic *Eco*RI endonuclease forms high-affinity (picomolar) complexes with cognate DNA at pH 7.5 (*2*). Results with *Eco*RV endonuclease have been more variable: Depending on the conditions and methods used, cognate DNA dissociation constants in the absence of metal ions range from picomolar to nanomolar, with metal ions enhancing affinity 4−10000-fold (*3*, *4*). The apparent sensitivity of *Eco*RV behavior to experimental conditions and approaches has led to contentious discussion regarding the role of metal ions in DNA affinity and specificity of restriction enzymes. Indeed, the possibility that this dependence may indeed vary among restriction enzymes has been debated (*3*).

In an effort to lend some perspective to restriction enzyme structure−function relationships, we have undertaken a study of the representative *Pvu*II endonuclease, which cleaves at 5′-CAG|CTG-3′ sites in a Mg(II)-dependent manner to achieve blunt ends (*5*). In previous gel mobility shift studies, *Pvu*II endonuclease was found to depend strongly on the presence of divalent metal ions (Ca(II)) to form high-affinity complexes with DNA. Gel shifts by *Pvu*II endonuclease were not detectable with noncognate sequences nor in the absence of metal ions (*6*). Recently, we demonstrated using both calorimetry and ^{25}Mg NMR spectroscopy the presence of two metal ion binding sites per *Pvu*II active site (*7*). In addition to the catalytically supportive Mg(II), this result held for Ca(II) and Mn(II) (*8*), which support binding and DNA cleavage, respectively. The presence of two Ca(II) ions has been confirmed by crystallographic studies of a *Pvu*II endonuclease−cognate DNA complex (*9*). We now turn to dissecting how these metal ions contribute to function, using multiple binding assays to quantitate the contribution of divalent metal ions to DNA binding affinity and specificity.

MATERIALS AND METHODS

Materials. Nitrocellulose filters (0.2 μm pore size) were purchased from Schleicher and Schuell (Keene, NH). Chelex resin was purchased from Bio-Rad (Hercules, CA). Pura-

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tronic $MgCl_2$ and $CaCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). Concentrations of stock solutions were determined by flame atomic absorption spectroscopy using a Perkin-Elmer AAnalyst 700 spectrophotometer. All buffers were rendered metal free using Chelex resin prepared as described by the vendor and verified by atomic absorption spectroscopy (*10*).

Preparation of PvuII Endonucleases. Purification of enzyme was accomplished using phosphocellulose chromatography and heparin−Sepharose affinity chromatography as previously described (*11*). Proteins were concentrated using Amicon Centriprep and Centricon concentrators and rendered metal free via exhaustive dialysis against metal-free buffer (*12*). All enzymes were quantitated using $\epsilon_{280} = 36900\ M^{-1}\ cm^{-1}$ for the monomer subunit and subsequently expressed with respect to the dimer. Routine assays of activity were performed using HEX[1]-labeled duplexes and time-resolved fluorescence spectroscopy (*13*).

Preparation of Oligonucleotides. Unlabeled and HEX-labeled oligonucleotides 5′-TCTAGGCAGCTGCGGAG-3′ (featuring the cognate or specific recognition sequence) and 5′-TCTAGGCA**CG**TGCGGAG-3′ (representing a noncognate substrate) and their respective complements were purchased desalted from IDT Technologies (Coralville, IA) and purified by PAGE and Elutrap (Schleicher and Schuell, Keene, NH). DNA was quantitated using $\epsilon_{260} = 1.845 \times 10^5\ M^{-1}\ cm^{-1}$. This extinction coefficient was calculated by Oligo Analyzer 2.0 (www.idtdna.com) and is based on nearest neighbor effects. The contribution of the HEX dye to absorbance at 260 nm is within experimental error. All oligonucleotide concentrations are expressed with respect to the duplex. Duplexes were formed by heating to 95 °C a mixture of 1 equiv of the HEX-labeled strand with 1 equiv of the complementary strand and permitting the sample to cool to room temperature overnight. Duplex formation was confirmed by native PAGE analysis. Where appropriate, 17 pmol of duplex DNA was radiolabeled using [γ-^{32}P]ATP (33 pmol of a 6000 Ci/mmol stock) (NEN, Boston, MA) and polynucleotide kinase (1 unit) as per manufacturer's instructions (Promega, Madison, WI). 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. 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 (Bio-Rad, Hercules, CA). Arrays of binding reactions containing fixed concentrations of radiolabeled duplex and varying concentrations of enzyme were assembled in microtiter plates and incubated for at least 10 min at 25 °C. After equilibration, reactions were vacuum filtered through the Slot-Blot apparatus and washed with ≈200 μL of binding buffer and dried. 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 analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Areas of intensity were selected using the ImageQuant box tool. For consistency, all selected intensities for a particular image were kept the same size. Following background correction, intensities transferred into the Microsoft Excel file where they were normalized. Corrected intensity is defined as the signal intensity at 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. Data were then analyzed according to the isotherm:

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

where θ is fraction of duplex bound (i.e., retained on the filter), [E] is the total enzyme concentration, and K_a is the association constant. Reported values are the averages of at least three determinations, and the ranges of these values are reported in the tables as errors. Fits to isotherms had typical R values in excess of 0.99.

Fluorescence Spectroscopy. Fluorescence emission intensities were collected on an Aminco SLM 4800 spectrofluorometer equipped with a polarization assembly. The temperature was maintained with a thermostated compartment at 25 °C. HEX-labeled oligonucleotides were excited at 532 nm, and the resulting emission was passed through a 550 nm cutoff filter (Corning). The slit width was set to 4 mm. All samples were monitored with stirring using a nitric acid-cleaned quartz cuvette (NSG Scientific, Farmingdale, NY). Three readings were taken at all four combinations of vertical (v) and horizontal (h) polarizer settings using the L format, taken over a 10 s integration time, and averaged. Anisotropy values were calculated from the equation:

$$A = I_{vv} - GI_{vh}/(I_{vv} - 2GI_{vh}) \quad (2)$$

where I is the recorded intensity at any of the four polarizer configurations and $G = I_{hv}/I_{hh}$. Corrected anisotropy values (A) were then plotted versus enzyme concentration and the data fit to a binding isotherm to yield K_a (*14*). As with the filter binding experiments, all values reported represent averages of at least three determinations.

Competition Fluorescence Anisotropy. 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. The resulting data were fit to equations describing a competition experiment using Scientist software (MicroMath, Salt Lake City, UT) as previously described (*15*).

RESULTS

Cognate DNA Affinity in the Presence of Ca(II). Given the established sensitivity of DNA binding measurements to the conditions and methods employed, experimental approaches were of special consideration. Indeed, quantitating the effects of metal ions on driving DNA binding by restriction enzymes is particularly challenging. Depending on conditions, dissociation constants range from very tight (picomolar) to very weak (micromolar), both extremes of which can be difficult to measure. In approaching a comprehensive study of DNA binding by *Pvu*II endonuclease, we were interested in striking a balance between assays which would permit the measurement of both strong and weak binding and those which best reflect true equilibria.

Gel mobility shift assays of DNA binding are heavily utilized and indeed have been widely applied to restriction

[1] Abbreviations: HEX, hexachlorofluorescein; NCFB, nitrocellulose filter binding.

enzymes, including *Pvu*II endonuclease (*6*, *16*−*20*). However, since this method is sensitive to off-rates (*3*) and not all of the conditions in which we were interested were amenable to electrophoresis, we did not pursue the technique.

Although not, strictly speaking, an equilibrium method, the nitrocellulose filter binding (NCFB) assay exhibits high sensitivity and as such is particularly robust in the determination of very tight (picomolar) binding constants. It has been applied extensively in restriction enzyme DNA binding studies (*3*, *21*, *22*).

As a complement to this assay, we applied fluorescence anisotropy or polarization, a method which not only reflects true equilibria but also has proven useful in determining a wide range of binding constants. This method is sensitive to the change in tumbling behavior of a dye upon formation of a larger molecular weight complex and has been applied to a number of DNA binding proteins (*14*, *23*−*25*).

Since *Pvu*II endonuclease does not possess well-positioned Trp residues which often make intrinsic fluorescence spectroscopy feasible (*26*), it was necessary to work with oligonucleotides end labeled with well-behaved fluorescent dyes. The dye which afforded the best sensitivity over a wide variety of conditions was hexachlorofluorescein (HEX). Using a non-self-complementary cognate 16-mer duplex 5′-end labeled on both strands with HEX, it is possible to directly detect binding events using both intensity and anisotropy measurements at concentrations as low as 500 pM. As shown in Figure 1A, titration of enzyme into 500 pM HEX-labeled oligonucleotide duplex in the presence of 10 mM $CaCl_2$ at pH 7.5 yielded an essentially stoichiometric titration curve. This pattern indicates that, under these conditions, the dissociation constant (K_d) is well below 500 pM. This behavior was also observed in a recent fluorescence anisotropy study of Ca(II)-supported DNA binding by *Eco*RV endonuclease (*15*).

To provide quantitation of binding constants in this range, we turned to nitrocellulose filter binding. In the presence of Ca(II) at pH 7.5, this assay reproducibly yielded a binding affinity of 53 ± 10 pM for radiolabeled duplex containing the *Pvu*II recognition sequence (Figure 1B; Table 1). This result confirms the estimate of binding affinity obtained via fluorescence anisotropy and is consistent with the $K_{d,app}$ of 110 pM measured by gel mobility shift (*6*). Identical measurements with radiolabeled duplexes featuring the HEX dye produced results comparable to that of the unlabeled duplex, indicating that the HEX moiety does not appreciably influence the enzyme−DNA interaction.

With this information in hand, it was then possible to return to fluorescence anisotropy to obtain binding constants of an unlabeled duplex through competition experiments with the HEX-labeled oligonucleotide. As has been demonstrated previously *(14)*, these experiments permit a higher working concentration of labeled oligonucleotide than necessary for direct measurements. In the presence of Ca(II), titration of unlabeled duplex into a complex of enzyme and HEX-labeled duplex resulted in a loss of anisotropy (Figure 2). Mathematical fitting of the two equilibria (see Materials and Methods) produces a binding constant for the unlabeled duplex of 56 ± 22 pM. This agrees very well with the results obtained using nitrocellulose filter binding assays and suggests that both methods provide reliable measurements of DNA affinity.

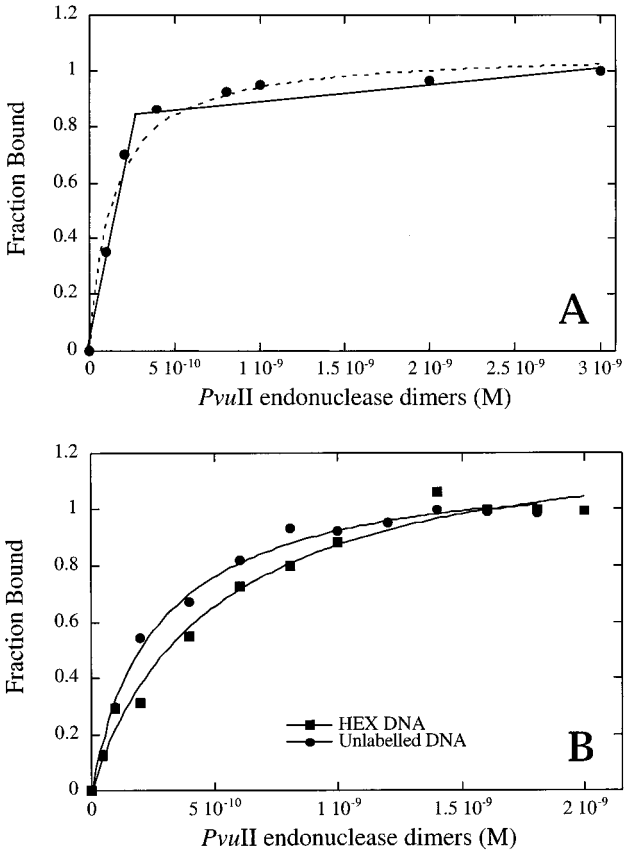

FIGURE 1: Cognate DNA binding by *Pvu*II endonuclease. (A) Titration monitored by fluorescence anisotropy at 500 pM HEX-labeled 16-mer duplex. The initial anisotropy value was 0.0705, and the final value was 0.127. The solid line is drawn to highlight the stoichiometric nature of the protein−DNA interaction at these concentrations. The dashed line represents the best fit to a binding isotherm. (B) Titration monitored by nitrocellulose filter binding at 25 pM duplex. Key: HEX-labeled (■) and unlabeled (●) oligonucleotide duplexes. Conditions: 50 mM Tris, 100 mM NaCl, and 10 mM $CaCl_2$, pH 7.5 at 25 °C.

DNA Binding under Metal-Free Conditions. To fully quantitate the effect of metal ions on restriction enzyme affinity for DNA, it is necessary to quantitate dissociation constants in the absence of metal ions. Such affinities can be very weak (micromolar), which necessitates the use of higher concentrations and methods which are well-suited to detect weak binding. Due to the wide concentration range with which experiments can be conducted, fluorescence anisotropy is an especially appropriate method. Also of concern is the rendering of metal-free conditions. In previous metal ion binding studies (*7*, *8*) we gained considerable experience in achieving metal-free conditions and therefore chose to approach these experiments in this fashion. As outlined in Materials and Methods, in addition to the liberal use of EDTA, care was taken to render buffers, enzyme, and DNA samples metal free using Chelex resin, exhaustive dialysis, and atomic absorption analysis. In our experience, unless handled properly, even quartz cuvettes can harbor metal ions and influence binding constants even when measurements are conducted in the presence of EDTA.

Under such scrupulous metal-free conditions, fluorescence anisotropy yields a *Pvu*II affinity of 307 ± 146 nM for the cognate sequence (Figure 3). This indicates that affinity for the cognate sequence is reduced ≈6000-fold when metal ions are absent.

Table 1: Summary of Sequence-Specific DNA Binding by *Pvu*II Endonuclease[a]

sequence	method	metal ions	dye	K_d
5′-TCTAGGCAGCTGCGGAG-3′ (cognate)	anisotropy	Ca(II)	HEX	<500 pM
		Ca(II)	none	56 ± 22 pM[b]
	filter binding	Ca(II)	none	53 ± 10 pM
		Ca(II)	HEX	55 ± 24 pM
	anisotropy	metal free	HEX	307 ± 146 nM
5′-TCTAGGCA**CG**TGCGGAG-3′ (noncognate)	anisotropy	Ca(II)	HEX	257 ± 60 nM
	filter binding	Ca(II)	none	204 ± 100 nM
	anisotropy	metal free	HEX	796 ± 50 nM
5′-TCTAGGGTCGACCGGAG-3′	anisotropy	Ca(II)	HEX	1.34 ± 0.19 μM[c]

[a] All values are averages of at least three measurements, and the corresponding errors reflect the ranges observed. Conditions: 50 mM Tris and 100 mM NaCl, pH 7.5 at 25 °C, unless otherwise indicated. Where used, the Ca(II) concentration was 10 mM. [b] Obtained in competition with HEX-labeled oligonucleotide duplex. [c] Collected at 87.5 mM NaCl.

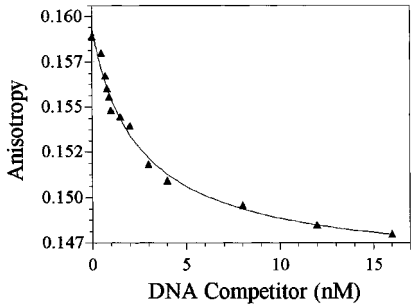

FIGURE 2: Cognate DNA binding by *Pvu*II endonuclease as measured by competition fluorescence anisotropy. Unlabeled 16-mer duplex was titrated into the 2.5 nM preformed HEX−duplex−enzyme complex. Conditions: 50 mM Tris, 100 mM NaCl, and 10 mM $CaCl_2$, pH 7.5 at 25 °C. The calculated K_d is 56 ± 22 pM for the cognate DNA sequence.

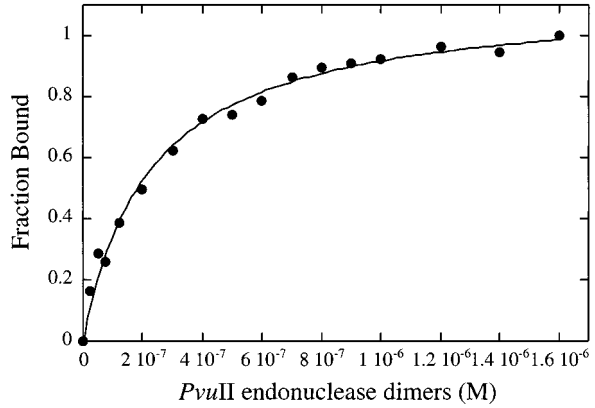

FIGURE 3: Cognate DNA binding by *Pvu*II endonuclease under metal-free conditions. The data were collected by fluorescence anisotropy with initial and final values of 0.0848 and 0.0924, respectively. Conditions: 50 nM DNA duplex, 50 mM Tris, 100 mM NaCl, and 3 mM EDTA, pH 7.5 at 25 °C.

Role of Metal Ions in Sequence Specificity. The ability to discriminate among DNA sequences is the hallmark of restriction enzyme function. The emerging trend for these systems is that metal ions play a role in sequence specificity (*4*, *15*, *27*). Indeed, for *Pvu*II endonuclease, Nastri et al. were unable to detect gel mobility shifts of nonspecific complexes in the absence of added Ca(II) (*6*). To quantitate the role of metal ions in *Pvu*II sequence specificity, measurements identical to those described above were conducted for a duplex in which the central GC step of the recognition sequence is replaced with CG (*6*). Regardless of whether Ca(II) is present or the experiment is conducted under metal-free conditions, the binding affinity for this duplex is in the high nanomolar range (Figure 4; Table 1). Similar results were obtained for the alternate noncognate sequence 5′-TCTAGGGTCGACCGGAG-3′. Thus it appears that (i) metal ions do not significantly influence affinity for noncognate sequences and (ii) metal ions are important to the discrimination between cognate and noncognate sequences by *Pvu*II endonuclease.

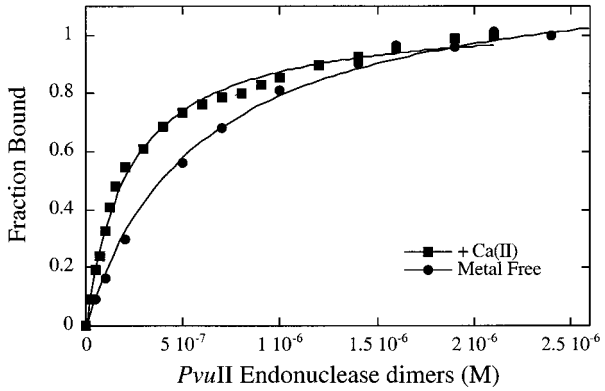

FIGURE 4: Noncognate DNA binding by *Pvu*II endonuclease as measured by fluorescence anisotropy at 50 nM 16-mer duplex (■) in the presence of 10 mM $CaCl_2$ and (●) under metal-free conditions in the presence of 3 mM EDTA. Initial and final anisotropy values were 0.04920, 0.05395 and 0.04865, 0.05360, respectively. Conditions: 50 mM Tris and 100 mM NaCl, pH 7.5 at 25 °C.

Ionic Strength Dependence of DNA Binding. Measurements of the dependence of binding affinities as a function of ionic strength are widely used to characterize electrostatic differences between specific and nonspecific DNA binding interactions (*28*). To establish these relationships for *Pvu*II endonuclease and permit facile comparisons across different enzyme systems and experimental conditions, we measured binding constants over a range of ionic strength conditions for cognate and noncognate sequences in the presence of Ca(II) and under metal-free conditions. A summary of these experiments for the *Pvu*II endonuclease system appears in Figure 5. Since both buffer and $CaCl_2$ add appreciably to ionic strength and we wish to compare Ca(II)-supported vs metal-free behavior, data are plotted and analyzed with respect to ionic strength. Record analysis (*28*) of these data indicates that, in the presence of Ca(II), 8.1 ± 0.6 cations are released when *Pvu*II endonuclease binds its cognate sequence. For noncognate sequence binding, 13.8 ± 2.0 cations are released. Under metal-free conditions, 1.1 ± 0.2 cations are released upon cognate complex formation. Weak binding of noncognate sequences in the absence of metal ions made salt dependence studies prohibitive.

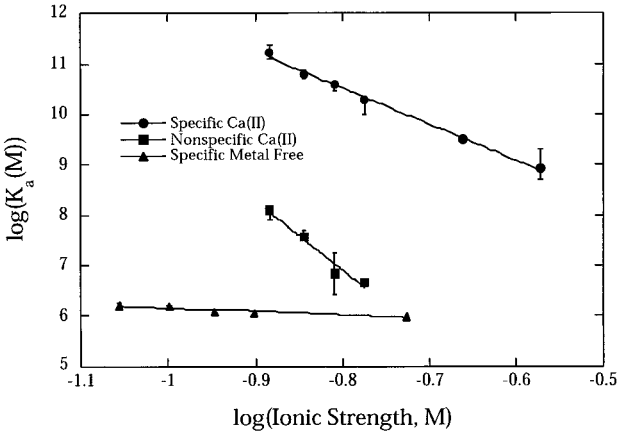

FIGURE 5: Ionic strength dependence of DNA binding by *Pvu*II endonuclease in 50 mM Tris, pH 7.5, as a function of NaCl concentration. Binding constants were obtained by either NCFB or fluorescence anisotropy. Ionic strength includes ionized buffer and $CaCl_2$ where appropriate. Key: (●) 16-mer containing the cognate sequence 5′-CAGCTG-3′ in the presence of 10 mM $CaCl_2$, as measured by NCFB; (■) 16-mer featuring the noncognate sequence 5′-CACGTG-3′ in the presence of 10 mM $CaCl_2$ as measured by fluorescence anisotropy; (▲) 16-mer containing the cognate sequence 5′-CAGCTG-3′ under metal-free conditions and 3 mM EDTA as measured by fluorescence anisotropy.

pH Dependence of DNA Binding. In an effort to understand the pH-dependent DNA binding behavior of *Pvu*II endonuclease and facilitate comparison to other enzymes, we measured *Pvu*II affinities for cognate and noncognate sequences in both the presence and absence of Ca(II) at a variety of pH values (Table 2). Measurements were made at pH 8 to explore behavior under widely utilized conditions for *Pvu*II cleavage; measurements were conducted at pH 7.5 to permit comparison to other studies. Since we observed a p$K_{a,app}$ of 6.7 for metal ion ligands in free *Pvu*II endonuclease (*7*), we were interested in whether this transition was reflected in DNA binding behavior. And finally, measurements at pH 4.5 are designed to explore DNA binding behavior under conditions typically used in the growth of crystals (*29*). To examine potential buffer effects, we measured, where possible, DNA binding constants in different buffers at the same pH. At pH 6 Tris, Bis-Tris propane, and succinate buffers gave similar binding constants. At pH 4.5, comparable binding constants were obtained in succinate and acetate buffers.

As summarized in Table 2, DNA binding of *Pvu*II cognate sites in the presence of Ca(II) exhibits a shallow pH dependence, becoming somewhat tighter at lower pH values. However, in the absence of metal ions, cognate DNA binding has no perceptible pH dependence. Similar behavior was observed for the noncognate sequence in both the presence and absence of metal ions.

DISCUSSION

Measurements of DNA Binding Affinity. In this study, we quantitate the effects of divalent metal ions on the DNA binding affinity and specificity of *Pvu*II endonuclease. Picomolar dissociation constants for DNA binding are not unusual among restriction enzymes; conversely, under less favorable conditions, high nanomolar to low micromolar K_ds are not unusual (*17*, *30*, *31*). Therefore, we approached the measurement of both strong and weak binding events by employing two complementary methods, nitrocellulose filter binding (NCFB) and fluorescence anisotropy. The former method has proven particularly useful in measuring very tight binding interactions (*3*, *22*). The latter is a spectroscopic method which detects bound species in true equilibrium with the free species. Because it is sensitive to the change in the tumbling rate of a fluorophore upon complex formation (*13*), this method is less susceptible to dye interference artifacts than intensity measurements. The inherent disadvantage is that, due to the use of the polarizers, most of the emitted light is not detected, which reduces sensitivity. This is of particular concern when measuring the equilibria of high-affinity complexes where concentrations are low. However, the application of competition experiments effectively addresses both dye interference and sensitivity issues. The use of multiple methods also serves as a means to verify measured binding constants. In a number of cases, similar binding constants were obtained with both NCFB and fluorescence anisotropy. This demonstrates that, through the use of these complementary methods, both high- and low-affinity DNA complexes of restriction endonucleases can be effectively and reliably quantitated.

Metal-Independent DNA Binding. Accumulating studies suggest that there is a range of restriction enzyme DNA binding affinities in the absence of metal ions. Classic studies of DNA binding by *Eco*RI endonuclease demonstrated that this enzyme forms high-affinity (picomolar) complexes in the absence of metal ions (*2*, *21*, *22*). Reports of an inability to observe weaker affinity DNA complexes of *Eco*RV endonuclease in the absence of metal ions (*16*) led to critical examination of means of measuring affinities and conditions employed. Using equilibrium and competition methods, more than one laboratory has been successful in quantitating the affinity of *Eco*RV endonuclease for its cognate sequence in the absence of added metal ions. Early studies reported very weak binding (K_d values in the micromolar range) (*17*, *31*). More recent studies reveal stronger binding; depending on conditions and the method used, log K_a ranges from 7 to 11 (*3*, *4*, *15*, *25*, *32*). DNA binding dissociation constants for *Bam*HI endonuclease are comparable (*27*). Table S1 (Supporting Information) features these examples and additional details for these and a number of other systems (*18*, *20*, *33*−*39*).

Although preliminary work by Nastri et al. foreshadowed weak binding by *Pvu*II endonuclease in the absence of metal ions (*6*), given the above reports, we were somewhat surprised that *Pvu*II cognate sequence dissociation constants under metal-free conditions were consistently above 200 nM, regardless of the conditions or method used. These values are appreciably weaker than that observed in many of the above studies and systems. It is difficult to ascertain the significance of this observation; it is possible that ion effects might be more pronounced for *Pvu*II endonuclease (*40*). Regardless, our results extend somewhat the range of expected behavior for metal ion-independent DNA binding by restriction enzymes.

Effect of Metal Ions on DNA Binding Affinity. The central motivation for these studies is quantitating the role of metal ions in driving restriction enzyme affinity for DNA. In an effort to put the effects of metal ions on DNA binding into perspective, we compiled available quantitative DNA binding data for restriction enzymes in an extensive table (Supporting

Table 2: pH Dependence of DNA Binding by *Pvu*II Endonuclease[a]

sequence	metal ions	K_d pH 8	pH 7.5	pH 6	pH 4.5
5′-TCTAGGCAGCTGCGGAG-3′ (cognate)	Ca(II)	62 ± 3 pM	53 ± 10 pM	15 ± 8 pM	18.6 pM
	metal free	492 ± 20 nM	307 ± 146 nM	357± 200 nM	230 ± 60 nM
5′-TCTAGGCA**CG**TGCGGAG-3′ (noncognate)	Ca(II)	323 ± 100 nM	257 ± 60 nM	251 ± 24 nM	1.01 ± 0.3 μM
	metal free	850 ± 50 nM	796 ± 50 nM	1.25 ± 0.1 μM	1.79 ± 0.4 μM

[a] All values are averages of at least three measurements, and the corresponding errors reflect the ranges observed. Conditions: 50 mM buffer and 100 mM NaCl, 25 °C.

Table 3: Summary of Recent Quantitative Measurements of the Metal Ion Dependence on DNA Binding by Restriction Enzymes[a]

enzyme	cognate Ca(II)/MF[b]	cognate Mg(II)/MF	noncognate Ca(II)/MF	noncognate Mg(II)/MF	ref
*Eco*RI	470	1		1	*3*, *21*, *27*
*Eco*RV (D90A)		4		10	*17*
*Eco*RV		∼10−100[c]		<0.001−0.0001	*31*
*Eco*RV (E45A/D74A/D90A)		30		0.0001	*31*
*Eco*RV	700[d]	4[d]			*3*
*Eco*RV	10000[d]	80[d]	1	1	*4*
*Eco*RV	≫30		3		*15*
*Bam*HI	240		1		*27*
*Pvu*II	6000		1		this study

[a] Entries are ratios of dissociation or association binding constants and are restricted to quantitative studies of systems involving determinations under at least two of the above conditions. For a more exhaustive survey, see Supporting Information. [b] MF = metal-free or in the absence of added metal ions. [c] Estimated from kinetic studies. [d] Measurements were made with modified oligonucleotides.

Table 4: Summary of Recent Quantitative Studies Comparing Cognate vs Noncognate Binding by Restriction Enzymes[a]

enzyme	K_d cognate/K_d noncognate Ca(II)	Mg(II)	metal free	ref
*Eco*RI			1000−7000	*21*
*Eco*RV			1	*17*
*Eco*RV (D90A)		1000	∼100	*17*
*Eco*RV		∼100000[c]	1	*31*
*Eco*RV (E45A/D74A/D90A)		1200000	10	*31*
*Eco*RV			5200/122[b]	*3*
*Eco*RV	10000	500	5	*4*
*Eco*RV (K38A)	35000	860		*4*
*Eco*RV	≫40		1−5	*15*
*Bam*HI	830000		3200	*27*
*Pvu*II	6000		1	this study

[a] Entries are ratios of dissociation or association binding constants and are restricted to quantitative studies of systems involving determinations under at least two of the above conditions. For a more exhaustive survey, see Supporting Information. [b] Measurements made at pH 6.25 and 7.0, respectively. All other measurements were made at or above pH 7.0. [c] Estimated from kinetic studies.

Information). Across multiple methods and under many solution conditions, many cognate DNA binding constants for restriction enzymes in the presence of Ca(II) are in the 10−100 pM range (*3*, *6*, *15*). To facilitiate comparisons to metal ion-independent binding, studies which involve quantitative measurements under metal free *and* in the presence of Ca(II) and/or Mg(II) are summarized in Tables 3 and 4. In those cases where comparisons can be made, one observes a uniform stimulation of cognate DNA binding in the presence of Ca(II) (Table 3). Using primarily filter binding techniques, the Jen-Jacobson group report factors of 470-fold for *Eco*RI endonuclease, 700-fold for *Eco*RV endonuclease, and most recently 240-fold for *Bam*HI endonuclease (*3*). Using gel mobility shift methods and a variety of conditions, Martin et al. report a 10000-fold increase in *Eco*RV cognate DNA binding affinity when Ca(II) is added (*4*). The behavior we observe for *Pvu*II endonuclease is similar to the latter *Eco*RV study: Comparing a 50 pM K_d in the presence of Ca(II) to the 300 nM K_d obtained under metal-free conditions yields a stimulation factor of 6000, a factor that falls within the reported range for restriction enzymes. Indeed, given the picomolar binding constant in the presence of Ca(II), this factor could be attributed to the weak interactions observed under metal-free conditions.

Studies of metal ion-dependent DNA binding are facilitated by the use of Ca(II), which supports binding but not turnover. There are a few studies which employ site-directed variants or substrate analogues to probe Mg(II)-driven DNA binding (*3*, *4*, *31*). Due to the compromises in enzyme or substrate structure necessary for such studies, comparison of Mg(II)- to Ca(II)-dependent binding can be problematic. However, it appears that, in those cases where data for both are presented in the same system, the stimulation of cognate DNA binding by Mg(II) appears somewhat less pronounced (Table 3). Additional studies would be helpful in determining if this is a general trend. While our efforts to measure DNA binding by *Pvu*II endonuclease in the presence of Mg(II) have thus far been unsuccessful, we remain interested in this issue and are continuing to pursue these measurements through a variety of techniques.

Sequence Specificity. Because affinities for different sequences are responsible for specificity, the involvement of metal ions in sequence specificity is also of interest. Nonspecific binding is uniformly weak among restriction enzymes, with many K_d values in the 100 nM range (Table S1, Supporting Information). These values reflect binding a few orders of magnitude weaker than that observed for cognate sequences, and the addition of Ca(II) does not significantly influence affinity for noncognate sites (Table 3) (*4*, *15*, *27*). As summarized in Tables 1 and 2, *Pvu*II endonuclease proves to be no exception to this trend,

exhibiting consistently weak affinities toward noncognate sequences across a wide variety of conditions.

With regard to specific, cognate sequence binding, a number of systems appear to depend on divalent cations for this behavior. Table S1 features examples of such systems. From this information we compiled Table 4, which for a number of restriction enzymes quantitatively compares cognate and noncognate affinities in the presence and absence of divalent cations. In those studies which involved both cognate and noncognate K_d measurements in the presence of Ca(II), there is consistent sequence discrimination; i.e., $K_{d,cognate}/K_{d,noncognate}$ is very large ($>10^3$). In the absence of added metal ions, reports of this ratio appear more variable. For three enzymes, the Jen-Jacobson group reports metal-free $K_{d,cognate}/K_{d,noncognate}$ values of 1000 or higher. The only exception is their report of a smaller ratio at pH 6.25 for *Eco*RV endonuclease (*3*). For other groups, most of which have studied this enzyme, the ratio is closer to unity. Collected over a wide variety of conditions using multiple methods, our data for *Pvu*II endonuclease are more consistent with the latter group, indicating that sequence discrimination is especially poor under metal-free conditions.

In an effort to further distinguish specific from nonspecific complexes, we determined the ionic strength dependences of the association constant for both types of complexes, measurements which are not typically reported for most systems. This effort constituted determinations conducted in both the presence and absence of Ca(II) and therefore necessitated normalization to ionic strength; the accompanying caveat is that ion-specific effects cannot be ruled out.

In the presence of Ca(II) we find that, in the physiological ionic strength range, noncognate sequence binding is more dependent on ionic strength than the specific complex formation. This behavior is consistent with that of other DNA binding proteins (*3*, *28*) and alludes to the thermodynamic difference between specific and nonspecific complexes. With regard to possible thermodynamic differences between Ca(II) driven and metal-free cognate complexes, our initial measurements reveal that the latter exhibits a very shallow ionic strength dependence up to 126 mM added NaCl, or 164 mM ionic strength. Such behavior has been reported for *Eco*RV endonuclease at slightly lower salt concentrations (<100 mM). Since it was possible that the ionic strength dependence of metal-free cognate DNA binding by *Pvu*II endonuclease is more dramatic at higher salt concentrations, we measured K_a at 150 mM added NaCl [188 mM ionic strength (Figure 5)] and found no dramatic change in the ionic strength dependence of the binding constant. Since we could not find any literature examples comparing the ionic strength dependence of metal-supported vs metal-free cognate DNA binding, it is difficult to know whether this is significant. It is possible that the ionic strength dependence of K_a becomes steeper at substantially higher salt, where in this case DNA binding is especially weak and difficult to measure ($K_d > 1\ \mu$M). Since the choice of salt can impact K_a (*28*), follow-up studies with other salts are planned.

Binding Affinity and Ionization Behavior. The pH dependence of DNA binding can provide important insights into the role of ionizable groups in DNA binding. The most systematic studies have been conducted for *Eco*RI (*2*), *Eco*RV, and *Bam*HI (*3*) endonucleases and their respective cognate sequences. In the absence of metal ions, all of these systems exhibit K_as at least 10-fold higher at pH 6 than at 7.5, with further increases in affinity at lower pH values. In contrast, *Pvu*II endonuclease exhibits little perceptible pH dependence of cognate DNA binding in the absence of metal ions, with K_ds remaining in the high nanomolar range throughout the entire pH range tested.

Little has been reported regarding the relationship between the pH dependence of DNA binding in the presence of metal ions. DNA binding studies of *Mun*I endonuclease and its active site variants reveal that cognate DNA binding is induced upon the addition of Ca(II) or a decrease in pH (*37*). The authors attribute this behavior to the anomalous ionization behavior of critical active site acidic groups which support DNA binding by either ligating Ca(II) in their ionized states or minimizing charge repulsion when protonated. Our recent ^{25}Mg NMR studies indicated an unusual p$K_{a,app}$ (6.7) for Mg(II) ligands in the free enzyme (*7*), a result consistent with interpretations of *Mun*I endonuclease behavior.

We were therefore especially interested in *Pvu*II cognate DNA binding behavior in the presence of Ca(II). Under these conditions, *Pvu*II cognate binding exhibits a shallow pH dependence, with affinity increasing about 4-fold between pH 8 and pH 4.5. Binding affinity remains in the picomolar range even at pH 4.5, where metal ion ligands would be protonated in the free enzyme. Thus it appears that Ca(II) exerts some effect on cognate affinity even at low pH. Additional experiments would be required to determine if this is due to differences in the pK_as of metal ion ligands in the presence of DNA relative to the free enzyme. In any event, the shallow pH dependence of DNA binding by *Pvu*II endonuclease most likely reflects the interplay between the protonation of ligands critical to metal ion-dependent binding and those which can compensate for the absence of metal ions. Indeed, the same groups could be responsible for both behaviors. The fact that pH-dependent effects are somewhat shallower than those observed for other restriction enzymes may simply reflect the collective effect of varied and multiple ionizations. In other words, a series of ionizations appropriately spaced throughout the physiological pH range could approximate an overall shallow pH dependence. Since it would be extremely difficult to measure individual pK_as in this system, we plan to further pursue this question using computational methods.

Conclusion. Data presented here reveal both similarities and differences between *Pvu*II endonuclease and other restriction enzymes. The use of multiple methods confirms that noncognate and metal-free DNA binding by *Pvu*II endonuclease is weak compared to many other systems. Further, substrate binding exhibits a remarkably shallow pH dependence relative to other characterized systems. However, when the effects of metal ions on DNA binding are comparatively considered, the increases in affinity and specificity observed upon the addition of metal ions are consistent with data reported for other systems, most notably *Eco*RV endonuclease. In this context, the apparently distinguishing behaviors of *Pvu*II endonuclease can be rationalized in terms of quantitative variability rather than alternate mechanisms of action. More generally, the data presented here indicate that, as with other restriction enzymes, *Pvu*II cognate affinity and sequence discrimination are most effectively achieved in the presence of divalent metal ions, illustrating the power of these cofactors in mediating protein−DNA interactions.

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SUPPORTING INFORMATION AVAILABLE

Table S1 showing an extensive tabulation of quantitative DNA binding measurements for restriction endonucleases. This material is available free of charge via the Internet at http://pubs.acs.org.

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