Investigation of Restriction Enzyme Cofactor Requirements: A Relationship between Metal Ion Properties and Sequence Specificity†

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ABSTRACT: Restriction enzymes are important model systems for understanding the mechanistic contributions of metal ions to nuclease activity. These systems are unique in that they combine distinct functions which have been shown to depend on metal ions: high-affinity DNA binding, sequence-specific recognition of DNA, and Mg(II)-dependent phosphodiester cleavage. While Ca(II) and Mn(II) are commonly used to promote DNA binding and cleavage, respectively, the metal ion properties that are critical to the support of these functions are not clear. To address this question, we assessed the abilities of a series of metal ions to promote DNA binding, sequence specificity, and cleavage in the representative *Pvu*II endonuclease. Among the metal ions tested [Ca(II), Sr(II), Ba(II), Eu(III), Tb(III), Cd(II), Mn(II), Co(II), and Zn(II)], only Mn(II) and Co(II) were similar enough to Mg(II) to support detectable cleavage activity. Interestingly, cofactor requirements for the support of DNA binding are much more permissive; the survey of DNA binding cofactors indicated that Cd(II) and the heavier and larger alkaline earth metal ions Sr(II) and Ba(II) were effective cofactors, stimulating DNA binding affinity 20−200-fold. Impressively, the trivalent lanthanides Tb(III) and Eu(III) promoted DNA binding as efficiently as Ca(II), corresponding to an increase in affinity over 1000-fold higher than that observed under metal-free conditions. The trend for DNA binding affinity supported by these ions suggests that ionic radius and charge are not critical to the promotion of DNA binding. To examine the role of metal ions in sequence discrimination, we determined specificity factors [K_a(specific)/K_a(nonspecific)] in the presence of Cd(II), Ba(II), and Tb(III). Most interestingly, all of these ions compromised sequence specificity to some degree compared to Ca(II), by either increased affinity for a noncognate sequence, decreased affinity for the cognate sequence, or both. These results suggest that while amino acid−base contacts are important for specificity, the properties of metal ion cofactors at the catalytic site are also critical for sequence discrimination. This insight is invaluable to our efforts to understand and subsequently design sequence-specific nucleases.

In the age of the genome, the enzymatic processing of nucleic acids has become an important research area. Many of these transformations, which include the synthesis, modification, repair, and degradation of all forms of RNA and DNA, involve metal ion cofactors. Correlated with its ubiquity and properties, Mg(II) remains the most commonly utilized cofactor in such transformations (*1*).

As we consider developing agents which can bind nucleic acids, perform sequence discrimination, and/or cleave these molecules, it becomes important to distinguish the properties of metal ions that support each of these distinct functions. Restriction enzymes provide unique molecular settings in which to examine this question. For a number of years, we have been pursuing structure−function relationships of type II restriction enzymes, homodimeric Mg(II)-dependent endonucleases which cleave DNA with remarkable sequence specificity (*2*). Relative to many nucleic acid enzymes, these systems are small, making them suitable for a wide variety of mechanistic and spectroscopic techniques. Metal ion cofactors support three distinct functions in restriction endonucleases: (i) They promote DNA binding affinity by these enzymes. Depending on the system and the metal ion, the increase in affinity over metal-free conditions is as high as 6000-fold (*3*). (ii) Additionally, through the modulation of binding affinities, metal ions help a number of these enzymes to discriminate cognate sequences from other sequences (*4*−*6*). (iii) Metal ions are absolutely critical to the phosphodiester hydrolysis reaction catalyzed by these enzymes (*2*).

Metal ion substitution is a longstanding strategy for understanding the important properties of metal ion cofactors. The extent to which alternative metal ions are tolerated by a given system provides valuable insights into what is required of these cofactors. Given the wide variety of size, charge, and coordination preferences, it is possible to clarify many features essential to the reaction in question. Despite this opportunity, very little information exists regarding how effectively various metal ions support the distinct functions of restriction endonucleases. Mn(II) generally supports cleavage activity in these enzymes (*7*); under some conditions, however, sequence specificity is compromised (*8*). Ca(II) is widely used in DNA binding studies of many nucleases because it promotes high-affinity DNA binding and sequence specificity without promoting cleavage (*6*, *9*, *10*). Beyond these few examples, little quantitative detail about the essential features of cofactor requirements exists.

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Over the past few years, we have focused on *Pvu*II endonuclease, a homodimeric type II restriction enzyme which cleaves at 5′-CAG|CTG-3′ to achieve blunt ends. This system has been quite amenable to a wide range of biophysical techniques. Applying isothermal titration calorimetry and ^{25}Mg NMR spectroscopy to Ca(II), Mn(II), and Mg(II), we determined that there are two metal ion binding sites per subunit and that these sites lie at the catalytic center (*11*, *12*). More recently, fluorescence anisotropy was applied to quantitate the metal ion dependence of DNA binding. Ca(II) stimulates cognate (specific) sequence binding by 6000-fold relative to metal-free conditions. This ion does not contribute to noncognate sequence (nonspecific) binding (*3*). Further, two metal ions per subunit participate in this process (*13*). These results are valuable because they provide important information about stoichiometry and the role of metal ions in achieving specific, high-affinity DNA binding; these pieces of information are critical to the validity of proposed mechanisms.

What these and other studies do not provide is broad information about the properties of the metal ions that are critical to supporting binding affinity, sequence specificity, and cleavage. As we continue to pursue knowledge of the nuclease mechanism, this issue has taken on new importance. Now that it has been established that quantitative binding and cleavage data can be obtained for this system over a wide range of conditions, we are now poised to apply this expertise to establish the importance of metal ion size, charge, and geometric preferences to supporting the distinct functions of restriction enzymes. In this study, the native Mg(II) cofactor is replaced with a series of metal ions which share some similarities to Mg(II) but also differ with respect to the aforementioned properties. The extent to which each metal ion supports DNA binding affinity, sequence specificity, and cleavage is quantitatively measured and used to assess the importance of each metal ion property to each endonuclease function. As an added incentive, this study also serves as a means for evaluating the potential for the use of appropriate metal ions as unique spectroscopic probes in ongoing mechanistic studies.

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). Puratronic $MgCl_2$, $BaCl_2$, $CaCl_2$, and $SrCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). $EuCl_3$ and $TbCl_3$ were purchased from Sigma Aldrich at 99.9% purity. All buffers were rendered metal free using Chelex resin prepared as described by the vendor and verified by atomic absorption spectroscopy (*14*).

Quantitation of Metal Ion Stocks. Concentrations of Mg(II) and Ca(II) stock solutions were determined by flame atomic absorption spectroscopy using a GBC model 904BT double beam atomic absorption spectrophotometer. Quantitation of Ba(II) and Sr(II) stocks was achieved by using Erichrome Black T indicator (*15*, *16*). Two hundred fifty microliters of ~10 mM Ba(II) or Sr(II) was added to a solution containing 25 mL of H_2O and 1 mL of NH_3-NH_4Cl, pH 10, buffer. Two hundred fifty microliters of 0.005 M Mg(II)-EDTA was added to provide a sharp end point. The subsequent addition of 3 drops of Erichrome Black T indicator produced a red solution. It is also important to add the buffer prior to addition of the indicator in order to prevent contaminating iron from reacting with the indicator. A standard solution of EDTA (10 mM) was titrated into the unknown solution until the end point (blue) was reached. Concentrations were determined from the equation:

$$[\text{unknown}]\,(\text{volume unknown}) = [\text{EDTA}]\,(\text{volume EDTA}) \quad (1)$$

where [unknown] is the concentration of the unknown metal stock, volume unknown = 250 μL, [EDTA] = 10 mM, and volume EDTA is the volume of the standard EDTA required to reach the end point.

The quantitation of the lanthanides was conducted as follows. Twenty-one microliters of 1 mg/mL Arsenazo III dye was added to 2.5 mL of 100 mM ammonium acetate, pH 7.1, producing a purple color. The unknown lanthanide was diluted to an approximate concentration of 10 mM, and 250 μL of this unknown was added to the above solution to produce a blue color. A standard solution of EDTA (10 mM) was titrated into a cuvette until the end point was reached (the solution returned to purple). Color transitions were monitored by collecting visible absorption spectra (300−700 nm) on a HP 8453 diode array spectrophotometer. Lanthanide (Ln) concentrations were determined from the equation:

$$[\text{Ln}]\,(\text{volume Ln}) = [\text{EDTA}]\,(\text{volume EDTA}) \quad (2)$$

where [Ln] is the concentration of the lanthanide, volume Ln = 250 μL, [EDTA] = 10 mM, and volume EDTA is the volume of the standard EDTA required to reach the end point (*17*).

Preparation of PvuII Endonucleases. The recombinant *Escherichia coli* expression system for wild-type and E68A *Pvu*II endonuclease was kindly provided by Dr. Paul Riggs of New England Biolabs. Purification of the enzyme was accomplished using phosphocellulose chromatography and heparin−Sepharose affinity chromatography as previously described (*18*). Proteins were concentrated using Amicon Centriprep and Centricon concentrators and rendered metal free via exhaustive dialysis against metal-free buffer (*19*). All enzymes were quantitated using $\epsilon_{280} = 36900\ M^{-1}\ cm^{-1}$ for the monomer subunit and subsequently expressed with respect to the dimer.

Preparation of Oligonucleotides. Unlabeled and HEX[1]-labeled oligonucleotides 5′-CAGGCAGCTGCGGA-3′ (featuring the cognate or specific recognition sequence) and 5′-CAGGCACGTGCGGA-3′ (representing a noncognate or nonspecific sequence) 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 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. Duplex formation was confirmed by native PAGE analysis. Samples

[1] Abbreviation: HEX, hexachlorofluorescein.

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 [γ-^{32}P]ATP (33 pmol 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. 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 425 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 $\approx$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 the boxes for all selected intensities for a particular image were kept the same size. Following background correction, intensities were transferred into a Microsoft Excel file and normalized. 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-end-labeled 14mer duplex DNA was incubated with 625 nM *Pvu*II endonuclease dimers in 50 mM Tris and 100 mM NaCl, pH 7.5, at 37 °C. Each reaction was initiated by addition of metal ion. At the indicated time, the reaction was quenched with an equal volume of 250 mM EDTA in 50% glycerol. Products were then analyzed using a 20% polyacrylamide/8 M urea/0.5 × TBE gel with 0.5 × TBE as the running buffer. Results were visualized with a Storm phosphorimager.

Fluorescence Anisotropy. Fluorescence emission intensities were collected on a Fluorolog-3 (SPEX) spectrofluorometer equipped with a polarization assembly. The temperature was maintained at 25 °C with a thermostated cell holder. HEX-labeled oligonucleotides were excited at 540 nm, and the resulting emission was 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

$$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) \quad (3)$$

where I is the recorded intensity at the indicated polarizer orientations.

Determination of Equilibrium Constants. Equilibrium dissociation constants were determined in triplicate at 50 mM Tris and 100 mM NaCl, pH 7.5, at 25°C except where otherwise indicated. Equilibrium constants weak enough to be measured spectroscopically were determined with HEX-labeled 14mer duplex using fluorescence anisotropy. To minimize interference from buffer−metal ion interactions, 50 mM triethanolamine (TEA) buffer was used for determinations in the presence of Cd(II). For the same reason, 5 mM HEPES and additional NaCl to compensate for ionic strength were used when determining binding constants in the presence of Tb(III). Control isotherms obtained with Ca(II) in the presence of these alternative buffers were comparable to those determined with Tris buffer. An inner filter effect of Tb(III) absorption of HEX emission light was observed, but slit widths were adjusted appropriately and changes in anisotropy were attributed to the formation of enzyme−DNA complexes. The remaining binding constants were obtained via nitrocellulose filter binding.

As appropriate, corrected intensities or anisotropy values (A) were then plotted versus enzyme concentration and the data fit to a simple binding isotherm (*20*) using Kaleidagraph 3.5 (Synergy, Reading, PA):

$$\theta = \frac{K_a[E]}{1 + K_a[E]} \quad (4)$$

where θ is the fraction of duplex bound, [E] is the total enzyme concentration, and K_a is the association constant. K_ds reported are averages of at least three measurements.

RESULTS

In this study, metal ion substitution was utilized as a means of dissecting the essential properties of metal ion cofactors in the DNA binding, sequence specificity, and cleavage functions of *Pvu*II endonuclease. In the selection of potential cofactors, we were particularly interested in metal ions that share both similarities and differences with Mg(II), the native cofactor, and Ca(II), the most common substitute for Mg(II) in endonuclease−DNA binding reactions (*9*, *10*). We also had an additional motivation for identifying metal ions that could serve as new spectroscopic probes in ongoing structure−function studies of the representative *Pvu*II endonuclease.

The series of cofactors studied and their relevant properties are summarized in Table 1. Mn(II) shares a similar size and coordination preferences with Mg(II) and is known to support cleavage in nucleases (*8*, *18*, *21*). While the same is true to some extent for Co(II), support of DNA cleavage by this ion is less universal (*22*, *23*) but has been reported for a number of nucleases (*7*, *24*−*26*). Zn(II), which has been shown to support activity in other nuclease systems (*27*, *28*), is small like Mg(II) but of course has some ligand preference and geometric differences.

Since Ca(II) so avidly promotes DNA binding among nucleases, we reasoned that metal ions that can replace Ca(II) in proteins would be instructive. Among the transition metals, the soft Cd(II) ion often replaces Ca(II) in proteins (*29*) and has two magnetic isotopes suitable for NMR spectroscopic studies (*30*). The lanthanides Tb(III) and Eu(III) are large and trivalent and thus provide tests of size and charge requirements. Moreover, these ions are generally excellent Ca(II) substitutes in proteins and have luminescence proper-

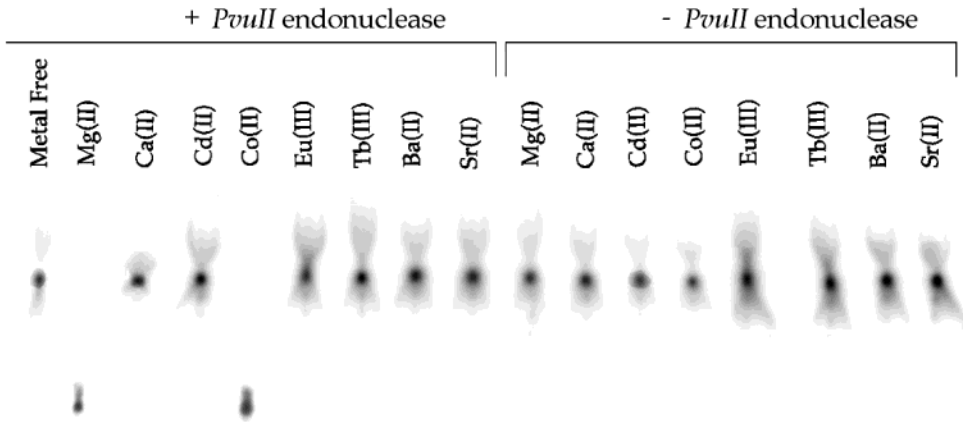

FIGURE 1: Denaturing PAGE analysis of *Pvu*II endonuclease−DNA cleavage reactions in the presence of various metal ions. Conditions: 125 nM duplex and 625 nM *Pvu*II endonuclease dimers in 50 mM Tris, 100 mM NaCl, and 10 mM metal ion at pH 7.5, 37 °C. The reaction time was 6 h. The Zn(II) reaction at 10 mM is not shown because it does not promote DNA binding under these conditions.

Table 1: Summary of Metal Ion Properties

	ionic radius, Å,[a] at coordination			
metal	6	7	8	log k_{ex},[c] s^{-1}
Mg(II)	0.86		1.03	5
Co(II)	0.88[b]		1.04	6
Zn(II)	0.88		1.04	7
Mn(II)	0.97[b]	1.04[b]	1.10	8
Tb(III)	1.06	1.12	1.18	8
Eu(III)	1.09	1.15	1.21	8
Cd(II)	1.09	1.17	1.24	8
Ca(II)	1.14	1.20	1.26	8
Sr(II)	1.32	1.35	1.40	9
Ba(II)	1.49	1.52	1.56	9

[a] Reference *78*. [b] High spin. [c] Exchange rates for metal-ligated water. Taken from ref *79*.

ties that permit the application of a number of useful spectroscopic techniques (*31*). Other larger alkaline earth metal ions such as Sr(II) and Ba(II) have been reported to support the activity of nucleases (*24*) and provide a means of evaluating the importance of size among divalent cofactors.

Support of DNA Cleavage by Metal Ion Cofactors. To determine to what extent any of the above metal ions promoted DNA cleavage by *Pvu*II endonuclease, we conducted long reactions (≥6 h), incubating the 125 nM ^{32}P-labeled 14mer oligonucleotide duplex containing the cognate sequence (see Materials and Methods) with an excess of enzyme (single-turnover conditions). The reactions were conducted at 10 mM, 1 mM, and 100 μM metal ions and subsequently quenched with EDTA; DNA products were evaluated via gel electrophoresis (Figure 1). Only Mg(II), Mn(II), and Co(II) promoted cleavage, even at high concentrations (500 nM duplex, 2.5 μM enzyme dimers) and long reaction times (22 h; data not shown). Under the stated reaction conditions, we did not observe cleavage at sites other than the target site. This held even for Mn(II) over multiple trials (data not shown).

Although cleavage was expected in the presence of Mn(II), Co(II)-supported cleavage is somewhat less common (*22*, *23*). To obtain quantitative information, time courses were conducted for single-turnover reactions in the presence of each of these metal ions. Densitometry of product bands yielded progress curves which were fit to an exponential to obtain k_{obs}. An example of such data appears in Figure 2, and single-turnover rate constants for Mg(II), Mn(II), and Co(II) are summarized in Table 2. Under these conditions, Mg(II) and Mn(II) supported rate constants of about 2 min^{-1}; Co(II) supported a rate approximately 10-fold slower.

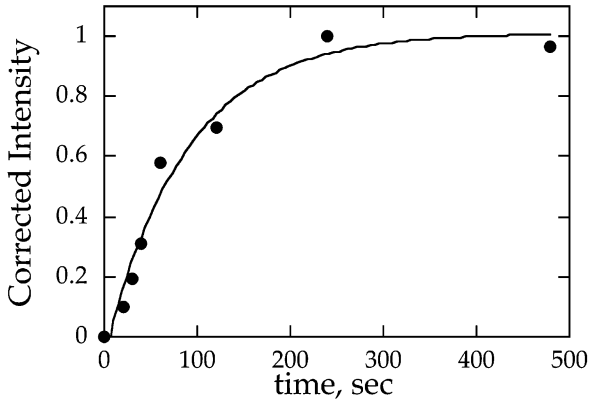

FIGURE 2: Single-turnover kinetic progress curve in the presence of Mg(II). Conditions: 125 nM 14mer duplex and 625 nM *Pvu*II endonuclease dimers in 10 mM $MgCl_2$ and 50 mM Tris, pH 7.5, 25 °C. Data were fit to a single exponential; k_{obs} = 0.68 min^{-1}.

Table 2: Single-Turnover Cleavage Rate Constants[a]

metal ion	k_{obs}, min^{-1}
Mg(II)	2.6 ± 2.0
Mn(II)	1.9 ± 1.4
Co(II)	0.17 ± 0.08

[a] Conditions: 125 nM 14mer duplex, 625 nM enzyme dimers, 50 mM Tris, 100 mM NaCl, and 10 mM metal ion at pH 7.5, 25 °C. Values are the average of at least three determinations.

Support of DNA Binding by Metal Ion Cofactors. The goal of these experiments is to determine if any of the above metal ions support the DNA binding function of *Pvu*II endonuclease. Initial measurements were made at 10 mM metal ion for a number of reasons: First, it is a typical metal ion concentration for assays and commercial buffers. Second, in our studies of the Ca(II) dependence of DNA binding by *Pvu*II endonuclease, we found that DNA binding constants were nearly constant between 5 and 10 mM metal ion (*13*). In addition, previous studies from this laboratory indicate that Ca(II), Mn(II), and Mg(II) bind the free enzyme with a dissociation constant of 1 mM (*11*, *12*), a value which is typical of nucleases (*32*−*34*). Finally, in the case of *Eco*RI endonuclease, kinetic cleavage data were fit to obtain a K_d

Table 3: Metal Ion Dependence of DNA Dissociation Constants[a]

metal	K_d, M	fold increase in K_a over metal free[b]	assay
Ca(II)			
10 mM	$(82 \pm 61) \times 10^{-12}$	6500	NCFB[c]
1 mM	$(244 \pm 162) \times 10^{-12}$	2200	NCFB
100 μM	$(36 \pm 5) \times 10^{-9}$		anisotropy
Tb(III)			
10 mM	$(56 \pm 11) \times 10^{-12}$	9500	NCFB
1 mM	$(267 \pm 236) \times 10^{-12}$	2000	NCFB
100 μM	$(617 \pm 140) \times 10^{-12}$		anisotropy
Eu(II)			
10 mM	$(116 \pm 45) \times 10^{-12}$	4600	NCFB
Sr(II)			
10 mM	$(3.1 \pm 1.6) \times 10^{-9}$	170	anisotropy
Ba(II)			
10 mM	$(22 \pm 8) \times 10^{-9}$	24	anisotropy
1 mM	$(10 \pm 8) \times 10^{-9}$		anisotropy
100 μM	$(4.4 \pm 1.0) \times 10^{-6}$		anisotropy
Cd(II)[d]			
10 mM	$(5 \pm 3) \times 10^{-9}$	100	anisotropy
1 mM	$(65 \pm 24) \times 10^{-9}$	8	anisotropy
Zn(II)			
10 mM	$(1.08 \pm 0.25) \times 10^{-6}$	1	anisotropy
1 mM	$(0.30 \pm 0.21) \times 10^{-6}$		anisotropy
metal free	$(0.53 \pm 0.39) \times 10^{-6}$		anisotropy
metal free + EDTA	$(4.8 \pm 2.9) \times 10^{-6}$		anisotropy

[a] All values are averages of at least three measurements. Conditions: except for otherwise noted, 50 mM Tris, 100 mM NaCl, and 10 mM metal ion at pH 7.5, 25 °C. All measurements made at concentrations lower than 10 mM metal ion were adjusted to constant ionic strength with the addition of an appropriate amount of NaCl. [b] Lower limit of the fold effect, based conservatively on the metal-free (no EDTA) conditions. [c] Nitrocellulose filter binding. [d] Measurements were conducted in 50 mM triethanolamine to minimize buffer−metal ion interactions.

of 3 mM for Mg(II) in the presence of DNA (*35*). This means that 10 mM metal ion would permit nearly complete occupancy of the complex.

Since we anticipated that this series of metal ions would support a wide range of binding constants, it was especially important to first characterize metal ion-independent binding for the 14mer oligonucleotide duplex used in these studies. Since metal-free binding is very weak (K_ds near 1 μM), these values were obtained using 5′-HEX-labeled oligonucleotides and fluorescence anisotropy. Two versions of this measurement were conducted: One binding constant was obtained in buffer rendered metal free using a Chelex column, and the other was obtained in the same system with EDTA added to 1 mM (Figure 3A). As summarized in Table 3, both K_ds were expectedly very weak (near 1 μM). Interestingly, we do observe an appreciable difference in values obtained in the presence and absence of EDTA. Whether this difference is due to the presence of adventitious metal ions below the limits of detection or due to an interaction between the enzyme and the polyanionic EDTA is not clear. When making comparisons between K_ds obtained in the absence and presence of various metal ions, we report a lower limit of the fold effect, based conservatively on the metal-free binding (no EDTA) binding constant.

Next, DNA binding constants were determined in the presence of 10 mM metal ion for all of the above metal ions. Due to the wide range of metal ions, their properties, and DNA binding affinities, it was necessary to apply two techniques: Nitrocellulose filter binding is useful in the measurement of very tight (picomolar) DNA binding constants (*36*, *37*); fluorescence anisotropy is more facile in the measurement of nanomolar to micromolar binding constants (*3*, *4*). The application ranges of the two techniques overlap in the low nanomolar regime. Previously, we established that equivalent DNA binding constants can be obtained for *Pvu*II endonuclease in the presence of Ca(II) by fluorescence anisotropy or nitrocellulose filter binding (*3*). As an example, at 0.5 mM $CaCl_2$, cognate DNA *Pvu*II dissociation constants obtained with nitrocellulose filter binding and fluorescence anisotropy were 9 and 12 nM, respectively.

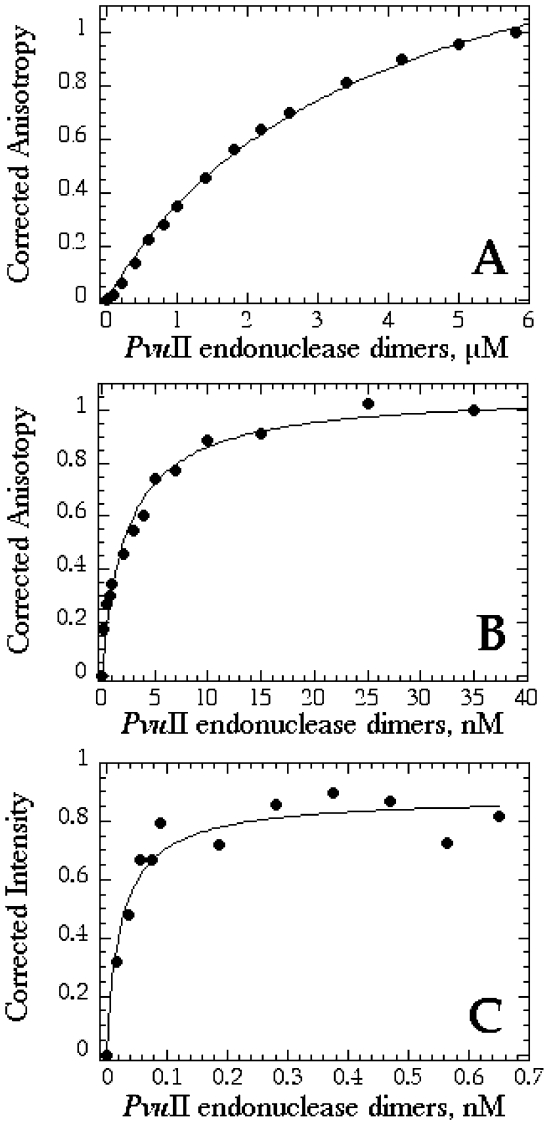

FIGURE 3: (A) DNA binding isotherm under metal-free conditions in the presence of 1 mM EDTA. The duplex concentration was 100 nM. The initial and final anisotropy values were 0.1097 and 0.1673, respectively. The application of the simple Langmuir isotherm and the Hill equation (*13*) yielded similar binding constants with similar residuals. K_d averaged from three curves is 4.8 ± 2.9 μM. (B) DNA binding isotherm in the presence of Ba(II) as measured by fluorescence anisotropy. The duplex concentration was 1 nM. The initial and final anisotropy values were 0.1427 and 0.2049, respectively. $K_d = 4.4 \pm 2.6$ nM. (C) DNA binding isotherm in the presence of Tb(III) as measured by nitrocellulose filter binding. The duplex concentration was ≈25 pM, and the data collectively fit to a K_d of 56 ± 11 pM. Conditions: 50 mM Tris, 100 mM NaCl, pH 7.5, 25 °C, and 10 mM metal ion except where otherwise noted. All values reported in the table are averages of at least three measurements.

For those metal ions which promoted nanomolar DNA binding affinity but did not possess interfering optical behavior [Cd(II), Zn(II), Sr(II), and Ba(II)], fluorescence anisotropy was applied. Figure 3B features a DNA binding curve obtained in the presence of Ba(II) via this technique. For those metal ions supporting picomolar affinity (which is difficult to measure optically) and/or absorbed or emitted light at wavelengths that interfered with the emission of HEX [Ca(II), Eu(III), and Tb(III)], nitrocellulose filter binding was used. No solubility problems were observed with the enzyme (picomolar to nanomolar range) at 10 mM lanthanide. Figure 3C illustrates a DNA binding isotherm obtained in the presence of Tb(III) using nitrocellulose filter binding.

Table 3 is a summary of *Pvu*II cognate DNA binding constants obtained in the presence of the various metal ions. All values are compared to binding constants obtained under scrupulously metal-free conditions. While there is an ionic strength difference between solutions containing metal ions and those which do not, the very shallow ionic strength dependence of metal-free DNA binding by *Pvu*II endonuclease makes comparisons facile (*3*). Binding constants obtained in the presence of Zn(II) were indistinguishable from those collected under metal-free conditions, indicating that this metal ion does not promote DNA binding. Cd(II), Ba(II), and Sr(II) each modestly stimulated DNA binding, supporting K_ds about 20-fold lower (tighter binding) than that observed under metal-free conditions. Most impressively, both Eu(III) and Tb(III) promoted DNA binding affinity by *Pvu*II endonuclease as efficiently as Ca(II), supporting picomolar dissociation constants. Thus it is clear that less stringent constraints are placed on metal ion properties in support of *Pvu*II DNA binding than cleavage (see Discussion).

The above interpretation rests on the assumption that DNA binding is not stronger at lower metal ion concentrations. Strictly speaking, this cannot be ruled out for some metal ions. It is possible that nonnative metal ion binding sites on the enzyme could inhibit DNA binding (*38*, *39*). To examine this possibility, we measured dissociation constants for DNA binding in the presence of 1 mM Ba(II), Cd(II), Zn(II), and Tb(III), a list that represents metal ions supporting DNA binding at both nanomolar and picomolar concentrations. As summarized in Table 3, for Ca(II), Tb(III), and Cd(II), binding is weakened approximately 10-fold relative to 10 mM metal ion at constant ionic strength. Only Zn(II), which has no discernible effect on DNA binding, and Ba(II), which supports similar K_ds at 10 and 1 mM metal ion, were exceptions to this trend. We reasoned that since binding constants at 1 mM metal ion were either comparable or weakened to that observed at 10 mM metal ion, it was extremely unlikely that binding would be stronger at 100 μM metal ion. Binding constants obtained in the presence of 100 μM Ca(II), Tb(III), and Ba(II) are consistent with this reasoning (Table 3).

Correlation of Metal Ion Binding Sites. While the above experiments identify which metal ions promote effective DNA binding, they do not address the issue of *where* these metal ions bind. Efficient DNA binding in the presence of these ions is consistent with active site locations for these ions. However, it is possible that positions in the active site could vary. Correlation of metal ion location with the extent of metal ion-promoted DNA binding would increase the interpretability of DNA binding experiments.

Table 4: Metal Ion Dependence of DNA Dissociation Constants for E68A[a]

metal	K_d, M	fold increase in K_a over metal free
Ca(II)		
10 mM	$(118 \pm 43.2) \times 10^{-9}$	16
1 mM	$(6.2 \pm 4.6) \times 10^{-6}$	1
Tb(III)[b]		
1 mM	$(136 \pm 58.5) \times 10^{-9}$	14
Ba(II)		
10 mM	$(390 \pm 184) \times 10^{-9}$	5
Cd(II)[c]		
1 mM	$(78 \pm 40) \times 10^{-9}$	24
metal free	$(1.9 \pm 0.8) \times 10^{-6}$	

[a] All values are averages of at least three measurements made using fluorescence anisotropy. Conditions: except for otherwise noted, 50 mM Tris, 100 mM NaCl, and 10 mM metal ion at pH 7.5, 25 °C. All measurements made at concentrations lower than 10 mM metal ion were adjusted to constant ionic strength with the addition of an appropriate amount of NaCl. [b] Measurements were conducted in 5 mM HEPES with compensating NaCl to minimize buffer−metal ion interactions. [c] Measurements were conducted in 50 mM triethanolamine to minimize buffer−metal ion interactions.

X-ray crystallography is the typical means by which metal ion locations are determined. However, such positions can be established thermodynamically by determining how effectively various metal ions promote DNA binding by an active site variant known to be deficient in metal ion binding. The ideal mutant enzyme for these studies is E68A. This *Pvu*II variant fails to induce a gel mobility shift of cognate DNA in the presence of Ca(II) (*9*). Using calorimetric and spectroscopic techniques, we previously established that E68A does not bind Mg(II), Mn(II), or Ca(II) to any significant extent; these data established the importance of Glu68 to the binding of 2 equiv of Mg(II), Mn(II), and Ca(II) in each subunit's active site (*11*, *12*). Crystallographic studies confirm the structural features of this arrangement for Ca(II) (*40*). In addition, we have applied NMR spectroscopy to establish that E68A is conformationally quite similar to the wild-type enzyme (*41*). This latter result indicates that the compromise in function in E68A is not due to significant changes in structure.

If DNA binding affinities by E68A in the presence of various metal ions are comparable to those obtained under metal-free conditions, then the importance of Glu68 to metal ion binding will be supported. Metal ions which coordinate Glu68 as a critical ligand likely bind in locations very similar to those occupied by Mg(II), Mn(II), and Ca(II).

Using fluorescence anisotropy, it was possible to quantitate weak DNA binding by E68A. DNA binding constants for this variant in the presence of various metal ions and under metal-free conditions are summarized in Table 4. For all metal ions ions examined [Ca(II), Tb(III), Ba(II), and Cd(II)], the removal of the Glu68 carboxyl group resulted in high nanomolar to low micromolar DNA dissociation constants, yielding K_ds much more similar to those obtained with wild-type under metal-free conditions than in the presence of metal ions (Table 2). The difference in the fold increase in K_a over metal-free conditions between E68A and the wild-type enzyme is dramatic for Ca(II) and Tb(III); the differences are more modest for Ba(II) and Cd(II). Thus Glu68 appears to be important to the binding of the former two ions, a result

Table 5: Nonspecific DNA Binding Constants for *Pvu*II Endonuclease[a]

metal	K_d, M	K_a(specific)/ K_a(nonspecific)[b]	$\Delta G°$, kcal/mol[c]
Ca(II)			
10 mM	$(128 \pm 4) \times 10^{-9}$	1600	−4.3
1 mM	$(390 \pm 75) \times 10^{-9}$	1600	−4.3
Tb(III)			
1 mM	$(9.5 \pm 1) \times 10^{-9}$	35	−2.1
Ba(II)			
10 mM	$(298 \pm 17) \times 10^{-9}$	10	−1.4
Cd(II)			
1 mM	$(47 \pm 15) \times 10^{-9}$	1	0

[a] All values are averages of at least three measurements. Conditions: except for otherwise noted, 50 mM Tris, 100 mM NaCl, and 10 mM metal ion at pH 7.5, 25 °C. Measurements for Cd(II) were conducted at 1 mM to circumvent solubility issues associated with the measurement. To minimize the effects of buffer−metal ion interactions, Cd(II) values were obtained in TEA buffer and Tb(III) values were obtained in 5 mM HEPES and 145 mM NaCl at pH 7.5, 25 °C. [b] Specificity factor calculated from data in Tables 3 and 4. [c] Energy change describing transfer of the enzyme from a noncognate sequence to a cognate sequence obtained from the equation $\Delta G° = -RT \ln[K_a(\text{specific})/K_a(\text{nonspecific})]$ (*42*).

which localizes metal ion binding to this residue (see Discussion).

Metal Ion Substitution and Sequence Specificity. As mentioned in the introduction, metal ions can significantly impact the sequence specificity of restriction enzymes (*3*, *6*). In the case of *PvuII* endonuclease in the presence of Ca(II), the difference in binding affinity between specific, cognate sequence binding and binding to other sequences is over 1000-fold (*3*). Indeed, binding constants obtained for a noncognate sequence in the presence of 10 mM Ca(II) and the cognate sequence under metal-free conditions are comparably weak (*3*). Thus it appears that Ca(II) selectively promotes affinity for the target sequence.

We reasoned that given the range of metal ion properties represented in the above ions, some metal ion cofactors might inhibit the enzyme's ability to discriminate among sequences. Correlating such behavior with the properties of the metal ion would be invaluable to understanding how metal ions contribute to this process. We therefore measured DNA binding constants for a noncognate oligonucleotide duplex in the presence of metal ions which do not support cleavage. The oligonucleotide used contained a sequence in which the center two bases are inverted (see Materials and Methods), a modification that results in a weak binding constant (high nanomolar) even in the presence of Ca(II) (*3*, *9*). Once again, a subset of metal ions were examined [Cd(II), Ba(II), and Tb(III)]. Where possible, metal ion concentrations were kept at 10 mM; enzyme solubility issues at high nanomolar to micromolar protein necessitated measurements at 1 mM for Cd(II) and Tb(III). Values obtained in the presence of Ca(II) at both concentrations serve as the basis for comparison. Table 5 features these measurements as well as the specificity factors, which are defined as the ratios of the cognate sequence binding constant to that obtained with a nonspecific duplex [K_a(specific)/K_a(nonspecific)] (*42*).

Interestingly, the specificity factors differ significantly among the metal ions tested. At 1 mM Cd(II), the enzyme binds noncognate and cognate sequences with comparable affinities, demonstrating no perceptible sequence discrimination under these conditions. While the enzyme binds the noncognate sequence similarly in the presence of either Ca(II) or Ba(II), the decreased affinity of the enzyme for the target sequence in the presence of Ba(II) results in a specificity factor of only 10 for the latter ion. Finally, 1 mM Tb(III) supported higher enzyme affinity for the noncognate sequence than that observed in the presence of 1 mM Ca(II). Thus while Tb(III) supports picomolar binding of the target sequence, the increased affinity for the noncognate sequence resulted in a much smaller specificity factor (35) than that observed in the presence of Ca(II).

DISCUSSION

Metal Ion Substitution in Nucleases. Metal ion substitution has proven to be a successful strategy for determining the properties of metal ion cofactors that are critical to metalloenzyme function (*1*, *43*, *44*). A classic example is carbonic anhydrase, in which a wide variety of ions were substituted for the native Zn(II) ion (*43*). Collectively, this work has provided important insights into interactions between the substrate and features of the active site. To perhaps a lesser degree, this also proves to be the case for metal-dependent nucleases. For example, substitutions with Mn(II), Co(II), and similar ions have been applied in spectroscopic, kinetic, and crystallographic studies of staphylococcal nuclease (*45*) and the Klenow fragment of DNA polymerase I (*27*).

The application of metal ion substitution among the Mg(II)-dependent type II restriction endonucleases has been more limited. The ability of Mn(II) to support DNA cleavage by restriction enzymes has been established for some time (*7*, *8*, *46*). Reports of the use of other substitutes for Mg(II) in cleavage reactions are few. Vipond et al. reported a qualitative trend that reflected the suitability of Mn(II) and Co(II) as hydrolytic cofactors for *Eco*RV and *Eco*RI endonucleases relative to Zn(II), Cd(II), Ni(II), and Ca(II), the presence of which stimulated residual to nondetectable specific activities (*7*). Indeed, Mn(II)-supported *Eco*RV endonuclease activity permitted more detailed studies (*21*, *39*). The cleavage data presented here are quite consistent with these results. However, in light of earlier *Eco*RV endonuclease studies (*39*), different relative *Pvu*II cleavage rates at other metal ion concentrations cannot be ruled out.

When considering the function of DNA *binding* (as opposed to cleavage), comparison of the abilities of various metal ions has been limited. The lack of information about how other ions promote DNA binding stems somewhat from the difficulties associated with forming dead-end complexes in the presence of metal ions which support cleavage [namely, Mg(II) and Mn(II)]. In these cases, compromises involving substrate analogues and/or site-directed variants were necessary. The use of Ca(II), which stimulates DNA binding but does not promote cleavage, became widespread when it became clear that a number of restriction enzymes required divalent metal ions to promote efficient DNA binding (*4*, *9*, *10*). Substitution with Ca(II) or Mn(II) is widely applied in crystallographic studies (*40*, *47*, *48*). Depending on the study and approach used, the degree to which Ca(II) stimulates DNA binding ranges from a fewfold to over 1000-fold relative to metal-free conditions (*4*, *10*, *49*). Studies of the well-characterized *Eco*RV endonuclease indicate that both Ca(II) and Mg(II) stimulate DNA binding to approximately the same extent (*10*, *49*). In any case, it is

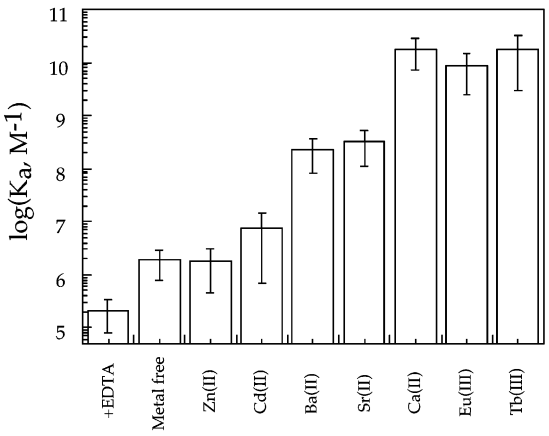

FIGURE 4: Summary of DNA binding affinities in the absence and presence of various metal ions. Conditions: 50 mM Tris, 100 mM NaCl, pH 7.5, 25 °C, and 10 mM metal ion.

clear that Ca(II) does support this function among type II restriction enzymes, as well as in a number of other nuclease systems (*50*, *51*).

Metal ion substitution has been more liberally applied to ribozyme systems. In these cases, a number of metal ions have been found to support cleavage. Hydrolysis rates for the hammerhead ribozyme in the presence Mn(II), Co(II), and Cd(II) are approximately 10-fold higher than that observed in the presence of Mg(II); even Ca(II) supports a residual activity (*52*). Aminoacyl transferase ribozyme and the group II intron ribozyme can cleave RNA in the presence of Tb(III) (*53*, *54*). Thus it appears that these nucleic acid systems appear to tolerate a wider variety of cations as cofactors than do their protein nuclease counterparts.

The unavoidable complication in the interpretation of all metal ion substitution experiments is that it is physically impossible to change one cofactor property (e.g., ionic radius) without also changing other properties (e.g., charge, ligand preference). It is therefore important to acknowledge that the behavior observed in metal ion-substituted enzymes is a function of all properties of the metal ion and how these properties alter the interaction of the metal ion with the enzyme and the DNA. Therefore, when making correlations between the behavior of enzyme complexes and metal ion properties, the effects of the cofactor substitution are correlated with a collection of inherent, associated properties of the cofactor. For example, the effects of larger cofactors almost certainly include slightly different positions in the active site relative to the placement of Mg(II). With these caveats in mind, the effects of metal ion substitution on DNA binding, cleavage, and sequence specificity are considered below.

Critical Cofactor Properties for DNA Binding. While Ca(II) clearly serves as an effective cofactor for DNA binding among nucleases, it alone does not provide sufficient information with which to identify the metal ion characteristics that are critical to DNA binding. Since so few of the metal ions surveyed support cleavage, an invaluable opportunity exists to use wild-type enzyme and native oligonucleotides to ascertain essential cofactor properties. With this goal in mind, we correlated the functions of *Pvu*II endonuclease with various metal ion properties (Table 1). Figure 4 graphically summarizes DNA binding affinities in the presence of various metal ions at 10 mM.

The formal charge on the metal ion cofactor does not appear to be critical to supporting DNA binding. The trivalent lanthanides promote DNA binding as efficiently as Ca(II). This observation suggests that electrostatics play a dominant role. Given the presence of a number of negatively charged groups in the active site (Asp58, Glu68, and Glu55 for *Pvu*II endonuclease), this seems reasonable and has been previously noted in restriction enzyme systems (*6*). Further support for electrostatic contributions to nuclease activity comes from a variant of RNase H, in which the conserved acidic groups that participate in Mg(II) binding are replaced with Arg residues with remarkable retention of function (*55*).

Tight DNA binding by *Pvu*II endonuclease also does not appear to depend strongly on atomic radius. Ions which are significantly larger than Mg(II) efficiently promote DNA binding, easily achieving dissociation constants in the picomolar range. We should clarify that comparisons between Mg(II) and other metal ions is still somewhat presumptive, since the degree to which Mg(II) stimulates *Pvu*II cognate DNA binding has not yet been quantitated. It is possible that Mg(II) does not stimulate DNA binding to nearly the same extent as Ca(II) or lanthanides and that picomolar affinity for DNA would compromise efficient cleavage. In vitro, one can envision a scenario in which the DNA association/dissociation rates (kinetic components of binding) could compromise effective turnover. Another possibility is that tight binding is inherently structurally different than weaker binding and the relevant groups are not optimally aligned, compromising cleavage rates. These possibilities are not universal, however: *Eco*RI endonuclease binds DNA avidly in the absence of metal ions, suggesting that picomolar DNA binding affinity is not inherently too tight for turnover (*56*). More complex scenarios emerge when considering conditions under which both Mg(II) and Ca(II) are present in high concentrations. In this case, if Ca(II) promotes DNA binding more efficiently than Mg(II), then it serves as competitive inhibitor, providing a means of control. On the other hand, if Mg(II) were to promote DNA binding as tight as Ca(II) and saturation conditions were maintained, Mg(II) could compete with Ca(II) effectively and cleavage would be more likely. Whether this is the case and if it makes an appreciable difference in the in vivo function of the enzyme remain to be determined.

Finally, the trend for DNA binding affinity supported by these ions suggests that a preference for oxygen ligands is crucial (Figure 4); softer ions which have a greater tolerance for nitrogen and/or sulfur ligands [Zn(II) and Cd(II)] promote *Pvu*II DNA binding much more weakly than the harder ions. This behavior provides an explanation for tight binding observed with Eu(III) and Tb(III); lanthanide complexes containing oxygen ligands are remarkably stable (*57*−*59*).

Thermodynamic Correlation of Metal Ion Binding Sites. In an attempt to localize metal ions to a specific ligand in the active site, DNA binding constants were measured for the variant E68A in the presence of a representative set of metal ions. By thus ascertaining the thermodynamic importance of Glu68 to metal ion binding, the proximity of the metal ion(s) to Glu68 is then deduced. A similar approach has been applied to assess metal ion locations in ribozymes (*60*).

The fold increase in K_a over metal-free conditions highlights the similarity of the binding constants obtained for E68A in both the presence and absence of metal ions. In

most cases, these values are smaller than those reported for the wild-type enzyme. The most dramatic differences in this factor between E68A and wild-type occur in the presence of Ca(II) and Tb(III), i.e., 1 vs 2200 for 1 mM Ca(II) and 14 vs 2000 for Tb(III), respectively. These differences implicate Glu68 as an important ligand for the binding of both these ions. This necessitates similar locations for these ions in the active site.

The differences between these factors for the enzymes are more modest for Ba(II) and Cd(II). In these cases, all factors are 20-fold or smaller. One could reasonably argue that these ions bind in positions that do not heavily involve ligand participation by Glu68. This provides additional rationalization as to why Ba(II) and Cd(II) promote DNA binding less effectively than Ca(II) or Tb(III): Due to differences in metal ion properties, they appear to bind in slightly different locations, which in turn compromises the efficiency of DNA binding.

Critical Cofactor Properties for DNA Cleavage. After consideration of the properties important to DNA *binding*, there remains the rationalization for why Mg(II), Mn(II), and Co(II) serve as cofactors in *hydrolysis* while the other metal ions tested do not. Metal ion properties can be critical to the optimal placement of ligands for catalysis. These metal ions are among the smallest tested, suggesting that ionic radius could also play a role. Larger ions may be accommodated in the active site and even promote DNA binding but may be located in slightly different positions in the active site. Further, the larger ions could likely place critical ligands in locations that are not conducive to hydrolysis. All three of these ions have some preference for octahedral geometry in the presence of oxygen ligands. This property is shared to some extent with the remaining metal ions, but Ca(II) and the lanthanides are more likely to coordinate more than six ligands (*61*). This tendency could generate some distortion of active site ligand placement, thus jeopardizing the delicate geometry needed for catalysis.

In addition to geometric considerations, dynamic characteristics may also be involved. Among the metal ions tested, Mg(II), Mn(II), and Co(II) have among the slowest water exchange rates (k_{ex}, Table 1). This property is often used as a gauge for rates of complex formation and/or ligand exchange. This is unlikely to be an issue with DNA binding, which has been shown to be virtually irreversible for *Pvu*II endonuclease in the presence of both Mg(II) and Ca(II) (*13*). However, since turnover numbers and cleavage rate constants for restriction enzymes are remarkably slow (1 s^{-1}) (*62*) relative to many enzymes (*63*), it may be detrimental to other aspects of the chemical mechanism. The other metal ions may simply exchange ligands too quickly.

Evaluation of Spectroscopic Probes. Part of our motivation for this study was to identify metal ion cofactors which could also serve as spectroscopic probes. In this respect, our study was also productive. Co(II) is useful in absorption and magnetic resonance spectroscopy (*43*). Thus spectroscopic techniques utilizing Co(II) may also prove informative; however, since Co(II) promotes cleavage by *Pvu*II endonuclease, the use of inactive enzyme variants and/or nonhydrolyzable substrate analogues would still be required.

In light of the favorable NMR properties of ^{113}Cd(II) (*30*), the stimulation of DNA binding by Cd(II) is encouraging. Even though the cognate DNA binding constant in the presence of Cd(II) is about 20-fold weaker than that observed with Ca(II), the thermodynamics favors the formation of enzyme−Cd(II)−DNA complexes at NMR concentrations. Depending on the goal of the experiment and metal ion affinities, enzyme solubility in the presence of Cd(II) could be a complication. However, if this issue can be adequately addressed, ^{113}Cd NMR experiments would be feasible.

From a spectroscopic standpoint, the most promising metal ions in the series tested were the lanthanide ions Tb(III) and Eu(III). Given their similarity to Ca(II), the ability of these ions to promote DNA binding in *Pvu*II endonuclease is not surprising. Lanthanide ions share with Ca(II) similar ionic radii, coordination geometries (6−8 ligands), water exchange rates, and a strong preference for oxygen ligands (Table 1; *57*−*59*). These similarities have led to extensive use of lanthanides and lanthanide spectroscopy in the study of Ca(II) binding proteins. This includes calmodulin (*64*), parvalbumin (*65*), protein kinase C (*66*), and the Klenow fragment (*67*). Such studies have been useful in identifying and characterizing metal ion binding sites (*31*). Thus, we are currently examining the potential of applying lanthanide spectroscopy to restriction enzymes. Since lanthanides typically bind metal ion-binding proteins with micromolar affinity or better (*61*), lanthanide concentrations can be kept under 1 mM, well below concentrations at which solubility difficulties arise.

Metal Ion Cofactors and Specificity. The involvement of metal ions in restriction enzyme sequence specificity became apparent in an early study of *Eco*RI endonuclease in which Mn(II) was substituted for Mg(II) (*8*). The observed relaxation of specificity, termed "star activity" (*68*), is usually manifested with large substrates in which a number of sites can be cleaved and thus easily detected by agarose gel electrophoresis. It can also be observed in the presence of high pH and/or in the presence of organic cosolvents (*68*, *69*). Given the premium placed upon sequence specificity in the design of new DNA ligands, the physical basis of this intriguing behavior is of general interest.

While some studies involving the effect of osmotic pressure on the sequence specificity of restriction enzymes have been conducted (*70*−*73*), to our knowledge there has been no thermodynamic analysis of the role of metal ions in this behavior. This study provides a new and unique opportunity to examine contributions of metal ions to sequence specificity. To this end, we utilized metal ions which promote substrate binding (but not cleavage) and the equilibrium DNA binding assay. The parameter of interest is the specificity factor, the ratio of the cognate sequence binding constant to that obtained with a noncognate sequence [K_a(specific)/K_a(nonspecific)] (*42*).

As a point of reference, the ability of Ca(II) to facilitate sequence discrimination at 10 mM has already been established for *Pvu*II endonuclease (*3*, *9*) and other restriction enzymes (*4*). While the duplexes used in these studies are shorter than those used for earlier *Pvu*II studies, the binding constants and hence the specificity factors are similar (3600). Of course, the observation of greater interest is the variable extent to which other metal ions contribute to sequence selectivity. Depending on the metal ion used, specificity factors range from 1 to 1600. Moreover, the mechanisms by which these specificity factors are achieved also vary. As highlighted in Figure 5, compromised specificity in the presence of Ba(II) stems from weakened binding to the

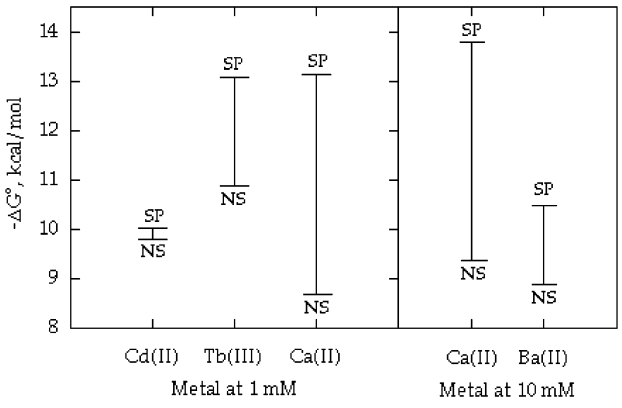

FIGURE 5: Binding energies for specific (SP) and nonspecific (NS) DNA binding by *Pvu*II endonuclease in the presence of metal ions. Data were collected at 1 mM metal ion to circumvent solubility issues with Cd(II) and Tb(III). Conditions are as described in Tables 3 and 5.

cognate sequence; nonspecific binding in the presence of this ion is quite similar to that observed with Ca(II). This results in an energy loss of about 3 kcal/mol relative to Ca(II)-stimulated DNA binding. An inverse pattern emerges for Tb(III); in this case, cognate sequence binding is as avid as it is in the presence of Ca(II); the difference lies instead in tighter binding of the nonspecific duplex. In this case, the remarkable affinities lanthanide ions have for oxygen ligands appear to be a distinct disadvantage, contributing to a specificity energy loss of about 2 kcal/mol relative to that observed with Ca(II) at the same concentration. The last permutation is illustrated with Cd(II). This ion has a severely compromised ability to promote discrimination not only because cognate binding is weak but also because nonspecific binding is also tighter, 10-fold better than Ca(II) under the same conditions. This combination of behaviors leaves no energetic difference with which to select sequences (i.e., $\Delta G^\circ \approx 0$).

What makes these observations so remarkable is that a simple change in metal ion cofactor can compromise specificity as efficiently as the mutation of single residues making specific base contacts with the cognate sequence. The best example for *Pvu*II endonuclease is the mutation of Asp34, a residue which makes a water-mediated contact to the internal guanine in the *Pvu*II recognition sequence (*74*). Upon substitution to Gly, sequence specificity is lost (*9*). Our studies show that substitution of Mg(II) with Cd(II) accomplishes the same thing for the wild-type enzyme. Further, while we cannot rule out allosteric binding sites for these ions, the fact that they promote DNA binding is consistent with active site location(s) for these ions. This would mean that determinants of specificity are not restricted to the region of the protein making sequence recognition contacts; these elements can also lie elsewhere, e.g., the catalytic site. This implies yet another form of strong coupling between sequence recognition and cleavage that is the hallmark of restriction enzymes. Indeed, the inverse of this relationship has already been established; substitution of residues involved in sequence recognition can have devastating effects on catalysis. D34G *Pvu*II endonuclease binds DNA but has no detectable cleavage activity (*9*). The same is true of H85A, another recognition site variant of *Pvu*II endonuclease (*9*), and Q115A, a similar variant in *Eco*RI endonuclease (*75*).

Given the obvious differences in sequence specificity supported by the metal ions tested, we reason that there must be a physical basis for this behavior. While it may be possible that different metal ions occupy site(s) on the enzyme with differing affinities, it seems unlikely that metal ion occupancy differences between specific and nonspecific complexes could be responsible for these behaviors. Another possibility lies in the enzyme's conformational response to these ions and/or in differences in how these ions are arranged at the active site. Indeed, we have observed enzyme conformational changes in the presence of Ca(II) that are not observed in the presence of Mg(II) (*18*). Crystallographic studies of *Pvu*II endonuclease indicate that the positions of various metal ions in the active site appear to be different (*40*, *76*). The same is true of related enzymes; the inactivity of the Eu(III)-substituted Klenow fragment was attributed to shifting of active site ligands relative to their placement in the presence of Zn(II) or Mg(II) (*77*).

The responses by the protein to metal ion binding described above remind us that the determinants of DNA binding, specificity, and cleavage by restriction enzymes lie not only in the metal ion but also in the enzyme and its conformational response to metal ion binding. In this respect, each enzyme−metal complex, in which both the enzyme and metal ion identities can be experimentally varied, has potentially unique means by which to accomplish these three tasks. Such complexities motivate studies of other restriction enzyme systems and their metal ion-driven behavior. The goal of such work would be to determine if the mechanisms of DNA binding, specificity, and cleavage are fundamentally similar across the systems or if there are multiple means of accomplishing these common functions. Such work, particularly in the areas of DNA binding and cleavage steps of the reaction, is ongoing (*13*, *39*, *49*).

One clear conclusion of these studies is that, at least for *Pvu*II endonuclease and the metal ions tested, Ca(II) is especially well suited to promote avid cognate sequence binding and effective sequence discrimination. Since such specificity is also observed in cleavage experiments with Mg(II) (*9*), clearly ionic radius is not the only important feature to this function. Given the observed difference in this behavior in the presence of Ca(II) and Tb(III), other factors such as charge, coordination number, and affinity for oxygen ligands are also likely important factors in this process. For these reasons, we remain interested in *Pvu*II DNA binding and sequence discrimination in the presence of cleavage-promoting metal ions Mg(II), Mn(II), and Co(II).

In summary, we have applied metal ion substitution to identify the properties of metal ion cofactors that are critical to the ability of *Pvu*II endonuclease to perform the distinct functions of sequence recognition, substrate binding, and cleavage. This study indicates that different combinations of metal ion properties are needed for each restriction enzyme function and provides new insights into the unique suitability of Ca(II) as a cofactor analogue of Mg(II).

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