A Catalytically Deficient Active Site Variant of *Pvu*II Endonuclease Binds Mg(II) Ions†

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ABSTRACT: In efforts to understand the mechanisms of many nucleic acid enzymes, the first site-directed mutations are made at conserved acidic active residues. Almost without exception, the low or null activities of the resulting variants are attributed to the importance of the acidic residue(s) to the ligation of required metal ions. Using ^{25}Mg NMR spectroscopy as a direct probe of metal ion binding and the homodimeric *Pvu*II restriction endonuclease as a model system, this interpretation is examined and clarified. Our results indicate that Mg(II) binds wild-type *Pvu*II endonuclease in the absence of DNA with a $K_{d,app}$ of 1.9 mM. Hill analysis yields an n_H coefficient of 1.4, a value consistent with the binding of more than one Mg(II) ion per monomer active site. Variable pH studies indicate that two ionizable groups are responsible for Mg(II) binding by wild-type *Pvu*II endonuclease near physiological pH. The p$K_{a,app}$ for these ionizations is 6.7, a value which is unusual for acidic residues but consistent with data obtained for critical groups in *Mun*I endonuclease and a number of other hydrolases. To assign residues critical to ligating Mg(II), binding measurements were performed on the low activity catalytic site mutants E68A and D58A. As expected, E68A binds Mg(II) ions very weakly ($K_{d,app} \approx 40$ mM), implicating Glu68 as critical to Mg(II) binding. Interestingly, while D58A has only residual specific activity, it retains an affinity for Mg(II) with a $K_{d,app}$ of 3.6 mM and exhibits a Hill coefficient of 0.7. Moreover, in this variant, multiple ionizable groups with p$K_{a,app}$ of 7.2 are involved in Mg(II) binding, suggesting a shuffling of Mg(II) ligands in the active site. These data indicate that Asp58 is important for the critical positioning of metal ion(s) required for catalysis.

Many Mg(II)-dependent nucleic acid enzymes feature a number of conserved acidic residues in their sequences. These residues are the centerpieces for a number of motifs, including the DDE motif of recombination enzymes (*1*), the LAGLIDAG motif of the homing endonucleases (*2*), and the PD...(D/E)XK motif of type II restriction endonucleases (*3*). X-ray crystal structures of these enzymes place these residues in respective catalytic sites (*4*−*6*). Further support for the importance of these residues to enzyme function are a number of site-directed mutagenesis studies in which one or more of these acidic residues are replaced with dissimilar residues (Ala, Gly, and Lys). The resulting variants often exhibit very low or undetectable specific activities (*7*−*10*). Without exception, these losses in activity are attributed to a loss of critical metal ion binding and have led to the assignment of these residues as Mg(II) ligands.

Perhaps due to the straightforward and attractive nature of the interpretation, very few additional studies have been performed on these low activity catalytic site variants. Indeed, there are very few direct studies of Mg(II) metal ion affinities of wild-type nucleic acid enzymes (*11*, *12*), much less on site-directed mutants. To our knowledge, no crystallographic studies have been performed on these variants. Thus, it is unclear whether these variants bind and/or orient Mg(II) or if their conformational behaviors are distinct from their respective native enzymes. Assignments of metal ion affinity to particular residues would be invaluable to debates of nucleic acid enzyme mechanisms.

Other factors limiting the examination of these questions are the difficulties associated with direct measurements of Mg(II) binding. Magnesium has no d shell electrons and is therefore not amenable to spectroscopic methods that rely on electronic transitions. Studies of ternary complexes with substrate are complicated by turnover and competitive metal ion binding by DNA (*13*). Unlike metal ion binding to other metalloenzymes (*14*), affinity for Mg(II) is often weak, which is consistent with high intracellular Mg(II) concentrations (*15*) but makes binding measurements very challenging.

A number of these difficulties can be overcome with the application of ^{25}Mg NMR spectroscopy, which directly measures macromolecular interactions with Mg(II) through spectral line shapes and chemical exchange between populations of free and bound metal ions (*16*). Especially suited to measure weak metal ion binding (millimolar range), this methodology has been successfully applied to a number of systems including calmodulin (*17*), tRNAPhe (*18*), RNase H (*11*), isocitrate dehydrogenase (*19*), yeast enolase, and pyruvate kinase (*20*).

There have been a number of mutagenesis and crystallographic studies of conserved acidic residues and metal ion binding in restriction enzymes (*12*). Due to their abundance,

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compact structures, and level of characterization (*3*), these nucleic acid enzymes are ideal systems for ^{25}Mg NMR spectroscopic studies. We have been developing the representative 2 × 18 kD *Pvu*II endonuclease as a model system (*12*, *21*). Like all known type II restriction endonucleases, this enzyme features conserved acidic (Glu68, Asp58, and Glu55) and lysine (Lys70) residues in each monomer active site. Mutagenesis studies have implicated Asp58 and Glu68 of *Pvu*II endonuclease as important to metal ion binding (*10*). Here, ^{25}Mg NMR spectroscopy is applied to determine the number, identities, and p$K_{a,app}$s of ionizable groups critical to Mg(II) binding in *Pvu*II endonuclease.

MATERIALS AND METHODS

Materials. A total of 99 atom % D D_2O and Tris were obtained from Cambridge Isotopes (Andover, MA). A total of 99.1% ^{25}MgO was purchased from Trace Scientific International (Toronto). ^{25}Mg(II) stock solutions were prepared by dissolving ^{25}MgO into dilute $HClO_4$, adding Tris to 2 mM to slow pH drift, and adjusting the pH to 7.7* with dilute hydroxide. Concentrations of stock solutions were determined by flame atomic absorption spectroscopy using a Perkin-Elmer 2380 spectrophotometer. Puratronic $CaCl_2$ was purchased from Alfa Aesar (Ward Hill, MA).

Preparation of PvuII Endonucleases. Preparation and purification of both wild-type and mutant *Pvu*II endonucleases were accomplished using phosphocellulose chromatography and heparin sepharose affinity chromatography as previously described (*12*). Pure fractions were pooled, dialyzed against the appropriate buffer, and concentrated using Amicon Centriprep and Centricon concentrators. Enzyme monomers were quantitated using $\epsilon_{280} = 36\,900\ M^{-1}\ cm^{-1}$ (*22*). Enzyme concentrations are expressed with respect to the subunit (monomer).

Activity Assays. Specific activities of NMR samples were determined using agarose gel electrophoresis of reaction products as described (*10*). Assays of mutant enzymes were conducted on 0.025 μg/μL λ DNA in 10 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, pH 7.9 (NEB buffer 2) at 37 °C. Reactions were initiated with the addition of enzyme and quenched with EDTA. Reaction times were kept short (<20 min), with convenient rates modulated with enzyme concentration (typically 1−280 nM monomers).

^{25}Mg NMR Spectroscopy. NMR samples typically contained 250 μM *Pvu*II monomers in 50 mM Tris, 400 mM KCl, pH* 7.7 in 10% D_2O at 25 °C. These conditions minimize aggregation and maximize enzyme solubility and stability during the experiment, as well as facilitate comparisons to earlier measurements (*12*). ^{25}Mg NMR spectra were collected on a Varian XL-400 spectrometer using a 10 mm, 9−30 MHz broad band probe tuned to 25 MHz. Since the spectra were not affected by acoustic ringing, a standard single pulse experiment was sufficient for data acquisition. Typical spectra were collected with 20 000 scans of 8192 points and processed with 10 Hz of line broadening. Line widths were determined using vnmr software (version 5.1a). Given the width of the resonance line and shimming efforts, we estimate that line width values are reliable to a few tenths of hertz. Most titrations spanned 15−35 Hz, leaving ample dynamic range to observe meaningful titration points.

^{25}Mg line width data obtained during binding experiments were fit to a modified version of the Langmuir isotherm in which the fraction of free enzyme θ_f is expressed in terms of the free metal ion concentration $[M]_f$:

$$E + M \overset{K_{a,app}}{\rightleftharpoons} EM \quad (1)$$

$$K_{a,app} = [EM]/[E]_f[M]_f \quad (2)$$

$$\theta_f = \frac{[E]_f}{[E]_t} = \frac{[E]_f}{[E]_f + [EM]} = \frac{[E]_f}{[E]_f + K_{a,app}[E]_f[M]_f} = \frac{1}{1 + K_{a,app}[M]_f} \quad (3)$$

where $[E]_f$ is concentration of free enzyme, $[M]_f$ is free Mg(II), and $K_{a,app}$ is the apparent dissociation constant. Also applied to the data was a similarly modified form of the Hill equation (eq 4):

$$\theta_f = \frac{1}{1 + K_{a,app}[M]_f^{n_H}} \quad (4)$$

where n_H is the Hill coefficient.

θ_f in terms of measurable parameters is

$$\theta_f = \frac{\Delta\nu_{obs} - \Delta\nu_{free}}{\Delta\nu_{bound}} \quad (5)$$

where $\Delta\nu_{obs}$ is the observed line width at any given $[M]_t$, $\Delta\nu_{free}$ is the line width of both free Mg(II) and free enzyme (typically 14 Hz with 10 Hz line broadening), and $\Delta\nu_{bound}$ is the line width at which all of enzyme (E) is bound. The data for which the total Mg(II) ion concentration is approximately the same as concentration of free ligand (i.e., $[M]_f \approx [M]_t$) were fit to eqs 4 and 5 to yield $K_{a,app}$ and $\Delta\nu_{bound}$. KaleidaGraph (version 3.0.5) was used to fit all data.

Since the affinity of E68A for Mg(II) was insufficient for the above analysis, a $K_{a,app}$ value for this mutant enzyme was estimated by using the known $K_{a,app}$ values and conditions for wild-type and D58A samples to correlate an observed line width with a specific value for fraction bound. These data were then applied to experimental conditions for E68A to estimate fraction bound and thus $K_{a,app}$.

pH Measurements. Variable pH experiments were performed on samples containing 250 μM *Pvu*II monomers and 1 mM ^{25}Mg(II) in 2 mM triethanolamine and 400 mM KCl in 10% D_2O. pH* was adjusted with small aliquots of concentrated acetic acid and triethanolamine and recorded both before and after each experiment in a thermostated water bath set to the probe temperature, 25 °C.

Line width data were fit to a number of equilibrium expressions as a function of $[H^+]$ to yield the ionization constant(s) (p$K_{a,app}$) and the number of ionizable groups. Note that the experiment only probes those ionizations important for Mg(II) binding. From basic ionization equilibria in which up to three protons are added to the protein (the conjugate base, A^{n-}):

$$H^+ + A^{n-} \overset{K_{a1}}{\rightleftharpoons} HA^{n-1} + H^+ \overset{K_{a2}}{\rightleftharpoons} H_2A^{n-2} + H^+ \overset{K_{a3}}{\rightleftharpoons} H_3A^{n-3} \quad (6)$$

one can derive a family of equations describing equivalent, nonequivalent, and interacting ionization events. The single-

ionization model is represented by

$$\theta_{A,P} = \frac{K_a[H^+]}{1 + K_a[H^+]} \quad (7)$$

where $\theta_{A,P}$ is the fraction of A^{n-} species protonated and K_a is the ionization constant. For cases in which the order of $[H^+]$ exceeds one, a version of the Hill equation can be used:

$$\theta_{A,P} = \frac{K_a[H^+]^n}{1 + K_a[H^+]^n} \quad (8)$$

Data were also applied to alternate models in which two, nonequivalent, noninteracting ionizations (eq 9); two, equivalent, interacting (cooperative) ionizations (eq 10); and three nonequivalent, noninteracting ionizations are considered (eq 11):

$$\theta_{A,P} = \frac{K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2}{1 + K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2} \quad (9)$$

$$\theta_{A,P} = \frac{K_a[H^+] + K_a{}^2K_{12}[H^+]^2}{1 + K_a[H^+] + K_a{}^2K_{12}[H^+]^2} \quad (10)$$

$$\theta_{A,P} = \frac{K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2 + K_{a1}K_{a2}K_{a3}[H^+]^3}{1 + K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2 + K_{a1}K_{a2}K_{a3}[H^+]^3} \quad (11)$$

K_{a1}, K_{a2}, and K_{a3} are distinct ionization constants and K_{12} is the cooperativity factor in eq 10. For other useful discussions of fitting NMR data to obtain ionization constants, see Shrager et al. (*23*) and Markley (*24*).

RESULTS

^{25}Mg NMR Spectroscopy of PvuII Endonuclease. ^{25}Mg resonances exhibit very little chemical shift dispersion relative to line width (*17*). As a result, typically only one signal, exchange-broadened free Mg(II), is observed in protein-binding studies. The line width of the bound ion is typically too broad to be detected. As illustrated in Figure 1, resonances A and D, the ^{25}Mg resonance is significantly broadened by the presence of *Pvu*II endonuclease, an observation consistent with the exchange process and thus Mg(II) binding by the enzyme.

Ca(II) Competes with Mg(II) for Common Binding Site-(s). Because it promotes DNA binding but does not support turnover, Ca(II) is widely applied in DNA binding (*10*, *25*) and crystallographic (*26*) studies of nucleic acid enzymes. Given that Ca(II) is much larger than Mg(II) (ionic radius of 0.99 vs 0.65 Å) and has some slightly different geometric preferences (*15*), there is concern that Ca(II) and Mg(II) sites do not overlap. To address this issue, Ca(II) was titrated into a wild-type sample containing 1.5 mM ^{25}Mg(II). As illustrated in Figure 2, the addition of Ca(II) to 5 and then 10 mM (Figure 2, resonances B and C) narrows the resonance, releasing the Mg(II) from the enzyme. Thus Ca(II) competes with Mg(II) for exclusive site(s) on the enzyme.

Quantitation of Mg(II) Binding by PvuII Endonuclease. Titration of ^{25}Mg(II) into a sample of *Pvu*II endonuclease

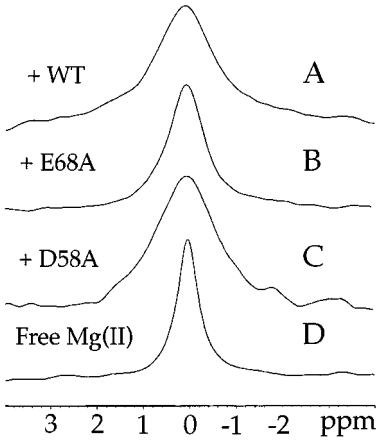

FIGURE 1: Mg(II) binding to *Pvu*II endonucleases as observed by ^{25}Mg NMR spectroscopy. All experiments were performed in 50 mM Tris, 400 mM KCl, pH 7.7* in 10% D_2O, at 25 °C. ^{25}Mg(II) (1 mM) in the presence of (A) wild-type, (B) E68A, (C) D58A, and (D) no enzyme.

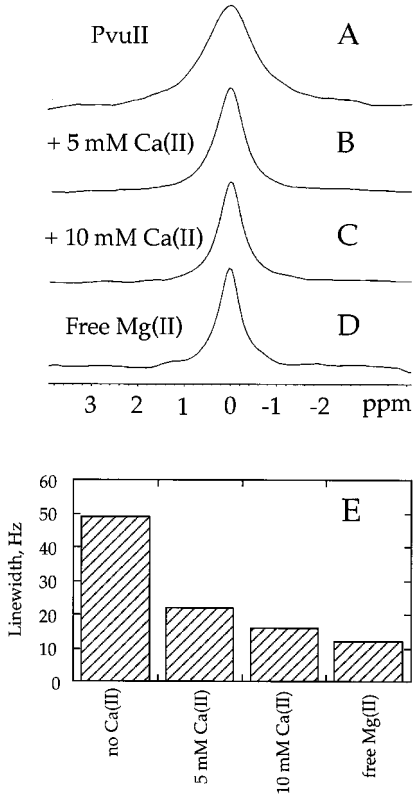

FIGURE 2: Competition between Mg(II) and Ca(II) for binding sites on *Pvu*II endonuclease. All experiments were performed with 250 μM *Pvu*II monomers in 50 mM Tris, 400 mM KCl, pH 7.7*, 10% D_2O at 25 °C. (A) ^{25}Mg(II) (1.5 mM); (B) 1.5 mM ^{25}Mg(II), 5 mM $CaCl_2$; (C) 1.5 mM ^{25}Mg(II), 10 mM $CaCl_2$; (D) free ^{25}Mg-(II). (E) Bar graph summarizing ^{25}Mg line width changes as a function of added $CaCl_2$.

enzyme results in a narrowing of line width as the enzyme becomes saturated and the fraction of the total metal ion bound to the enzyme decreases (Figure 3). Since the ^{25}Mg line width is proportional to the amount of enzyme−metal ion complex (EM) (*19*), it is possible to determine from these experiments an apparent dissociation constant ($K_{d,app}$) for Mg-(II) binding. Characteristically weak Mg(II) binding and the observation of large excesses of unbound (vs bound) metal ion do not permit direct determination of Mg(II)-binding

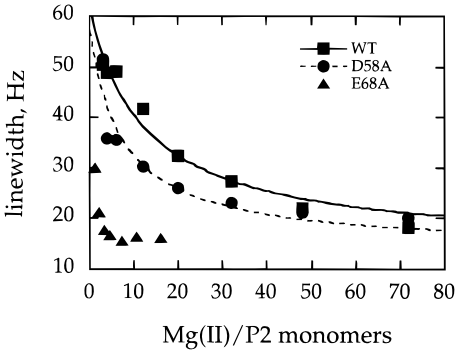

FIGURE 3: Titration of wild-type and mutant *Pvu*II endonucleases with ^{25}Mg(II). Sample conditions: 50 mM Tris, 400 mM KCl, pH 7.7* in 10% D_2O at 25 °C. Wild-type (■) and D58A (●) were collected at 250 μM *Pvu*II monomers; E68A (▲) data were collected at 750 μM *Pvu*II monomers.

Table 1: Summary of Mg(II) Binding Behavior by *Pvu*II Endonucleases[a]

enzyme	rel. specific activity	$K_{d,app}$ (mM)	R	n_H	R
wild-type	1.00	1.9 ± 0.4	0.9966	1.4	0.9980
D58A	0.0014	3.6 ± 0.5	0.9930	0.7	0.9987
E68A	0.026	~40	n/a	n/a	n/a

[a] Rates were determined in triplicate and are expressed as fractions of specific activity observed with wild-type enzyme. $K_{d,app}$ and n_H (Hill coefficient) were calculated or estimated as described in Materials and Methods.

stoichiometry by this method. Although there is some question as to whether there are two weakly cooperative metal ion binding sites per *Pvu*II subunit (see Discussion), for our current purposes these experimental data are satisfactorily represented by a simple Langmuir isotherm (Materials and Methods). Such an analysis yields a $K_{d,app}$ of 1.9 ± 0.4 mM for wild-type *Pvu*II endonuclease, comparable to K_ds for Mg(II) binding by other restriction enzymes obtained by other methods (*27*).

Evaluation of Active Site Groups as Mg(II) Ligands. Due to their conserved locations, acidic character, and the catalytic behavior of alanine-substituted *Pvu*II enzymes, Glu68 and Asp58 have been implicated as metal ion ligands (*10*, *28*). To localize Mg(II)-binding sites on the enzyme and to assess the contributions of these residues to metal ion binding, Ala mutant enzymes were assayed for both specific activity and Mg(II) binding. By modulating the enzyme concentration, it was possible to obtain specific activities for E68A and D58A (Table 1). As anticipated, both variants exhibited only residual catalytic activity (10^{-2} and 10^{-3} that of wild-type, respectively). In ^{25}Mg-binding experiments, exchange broadening is greatly reduced for samples containing E68A relative to wild-type, consistent with very poor affinity for Mg(II) (Figure 1, curves A and B). Indeed, the behavior of line widths as a function of Mg(II) concentration did not permit a quantitative fit of the data. Instead, binding affinity for this enzyme was estimated (Materials and Methods) to be ~40 mM (Table 1). In contrast to E68A, extensive exchange broadening of the ^{25}Mg resonance is observed in the presence of D58A (Figure 1C). Quantitative fitting of these data yield a $K_{d,app}$ of 3.6 ± 0.5 mM for this variant (Table 1). Thus, D58A has a nearly wild-type affinity for Mg(II).

pH Dependence of Mg(II) Binding. As pH is decreased, ionizable groups become protonated and less effective as metal ion ligands, which is in turn exhibited as resonance narrowing. By measuring the dependence of ^{25}Mg line width on pH, it is possible to determine the number of ionizable groups responsible for Mg(II) binding by wild-type *Pvu*II endonuclease. This experiment also provides a direct means of determining the p$K_{a,app}$(s) of ionizable groups responsible for metal ion binding.

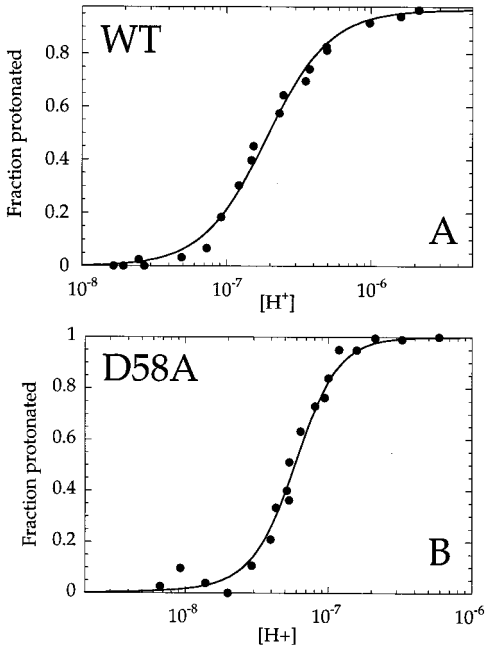

FIGURE 4: pH dependence of Mg(II) binding by wild-type (A) and D58A (B) *Pvu*II endonucleases. Sample conditions: 250 μM *Pvu*II monomers, 1 mM ^{25}Mg(II), 2 mM triethanolamine, 400 mM KCl, pH 7.7* in 10% D_2O at 25 °C. The p$K_{a,app}$ values of the ionizable groups responsible for metal ion binding were determined as described in Materials and Methods.

The ^{25}Mg concentration in this experiment (1 mM) was selected to provide the best signal-to-noise and the most sensitive line widths as the titration proceeds. A larger excess of free ^{25}Mg(II) restricts line width changes to a very small dynamic range and limits the interpretability of the data. As a consequence of the condition, the ionization of only those groups in the sites that are significantly occupied at 1 mM were probed in this experiment.

As shown in Figure 4A, Mg(II) binding to the wild-type enzyme drops precipitously between pH 6 and 7, with a p$K_{a,app}$ of 6.7 for ionizable groups responsible for metal ion binding. In an effort to determine if it is possible to dissect the number of ionizable groups and their respective pK_as, the data were fit to a number of ionization models. These include single, double, and triple ionization models involving equivalent, nonequivalent, and interacting (cooperative) models. To better determine the cooperativity factor (*29*), the two sites in the interacting model were constrained as equivalent. As judged by R values, fits to models involving two ionizable groups are clearly superior to those involving one or three ionizations (Table 2). A number of two-ionization models give fits of nearly equivalent quality and pK_a value(s) (see Discussion).

Since Mg(II) binding is so dramatically compromised in E68A, Glu68 must be one of the critical ligands of this cofactor. In an attempt to clarify the identity of the remaining ionizable group(s) involved in Mg(II) binding, an additional

Table 2: Summary of Mathematical Fitting of *Pvu*II Mg(II) Binding Ionization Behavior

			wild-type		D58A	
n^a	model	eq	pK_a	R	pK_a	R
1	single	7	6.6	0.9766	7.0	0.9374
2	equiv. independent.	8	6.7	0.9966	7.2	0.9853
float	equiv. independent	8	6.7, $n = 2.1^b$	0.9966	7.2, $n = 2.0^b$	0.9872
2	nonequiv. independent	9	5.7, 7.6	0.9954	5.7, 8.8	0.9804
2	equiv. interacting	10	6.1 (10)^c	0.9943	5.5 (3300)^c	0.9808
3	nonequiv. independent	11	6.2, 6.4, 6.5	0.9904	6.1, 7.0, 8.6	0.9934

[a] n = number of ionizing groups associated with Mg(II) binding. [b] Value of n to which the fit converged. [c] Cooperativity factor K_{12}.

pH titration was performed on D58A (Figure 4B). In this case, there is a perceptible shift in the apparent pH dependence of Mg(II) binding with a p$K_{a,app}$ of 7.2. These data fit best to a model of three nonequivalent independent sites with pK_as of 6.1, 7.0, and 8.6 (Table 2).

DISCUSSION

Probes of Metal Ion Binding. ^{25}Mg NMR spectroscopy has been applied to a number of metalloenzyme systems, typically with the goal of understanding affinity, relaxation, and exchange dynamics (*17*−*20*). In addition to providing direct measurements of Mg(II) affinity, these experiments also provide an opportunity to compare Mg(II)-binding behavior with that of Ca(II) and Mn(II). In nucleic acid enzymes, these nonnative divalent metal ions typically support DNA binding and turnover, respectively (*5*, *30*).

We recently applied both protein NMR spectroscopy and calorimetry to understand the metal ion binding behavior of *Pvu*II endonuclease (*12*, *21*). Interestingly, while both of these methods were sensitive to Ca(II) and Mn(II) binding, Mg(II) binding could not be detected, prompting speculation that this native metal ion does not bind the enzyme in the absence of DNA. The present study demonstrates that Mg(II) indeed does bind the enzyme in the absence of DNA. Moreover, the affinity of the enzyme for this metal ion is comparable to those obtained for Ca(II) and Mn(II) by calorimetry ($K_{d,app} \approx 1$ mM). In addition, the metal ion binding behaviors of active site-variants D58A and E68A are consistent: E68A exhibits little observable affinity for divalent metal ions, while D58A retains an ability to bind both Mn(II) and Mg(II). Thus, it appears that for *Pvu*II endonuclease, Mg(II) affinities of both wild-type and active-site variant *Pvu*II endonucleases parallel those obtained for Ca(II) and Mn(II) by other methods.

Crystallographic studies of a number of restriction enzyme−metal ion−substrate complexes consistently implicate the scissile phosphate of DNA substrates as a ligand of enzyme bound metal ions (*4*, *5*, *26*). This begs the question of how enzyme-bound DNA influences the affinity for Mg(II). One could certainly visualize a change in metal ion affinity accompanying the addition of the DNA phosphate ligand. There are a number of complications to performing this measurement, including the need to prevent turnover and minimize competitive Mg(II) binding by DNA. However, the most significant challenge is identifying a DNA that binds *Pvu*II endonuclease in solution in the absence of metal ions under experimental conditions. Without preformation of the ES complex, the interpretability of metal ion titration data in the presence of DNA is very limited.

pH Dependence of Mg(II) Binding. Unusual pK_a Values. The most obvious feature of the pH dependence data is the unusual p$K_{a,app}$ for Mg(II) binding. While the pK_as for free or exposed Asp or Glu are approximately 4.5, unusual pK_as for the acidic active-site residues of hydrolases have been reported (Table 3). Glu35 of lysozyme has a pK_a of 6.5 (*31*), and Glu172 of xylanase exhibits a pK_a of 6.8 (*32*). What makes the pK_a of these active-site groups unusually high? In both cases, mutagenesis studies have shown that other nearby acidic residues are responsible for the unusual pK_a values of these acidic groups (*32*, *33*). Conversely, nearby positive charges (Lys, for example) can depress acidic group pK_a values (*34*). Since there are both negatively and positively charged groups in nuclease active sites, it appears that one or more of these effects could be operative for *Pvu*II endonuclease.

Table 3: Unusual pK_as of Active Site Acidic Groups in Some Hydrolytic Enzymes

enzyme	group	pK_a	ref
carboxypeptidase A	Glu27	7.0	*52*
lysozyme	Glu35	6.5	*31*
xylanase	Glu172	6.8	*32*
*Mun*I endonuclease	Glu98, Asp83	$6.5 > x > 8.3$	*42*

Given this potential for complex behavior, rationalization of the basic shift in the pH dependence of Mg(II) binding by D58A is not straightforward. In theory, this could be caused by (i) an increase in pK_a for one or more existing ligands, (ii) the addition of a more basic group as a metal ion ligand, and/or (iii) the involvement of a basic group with a pK_a lowered into the experimental pH range. The pK_a of an acidic group would be lowered by the removal of an interacting acidic group (*32*, *33*). The influence of acidic groups on Lys ionization appears less straightforward. Theory predicts that Lys pK_a values are elevated by interacting acidic groups. This is attractive because removal of an interacting acidic group (Asp58 in this case) would indeed lower the pK_a of a Lys side chain (e.g., Lys70), bringing it closer to the transition region and more likely to influence p$K_{a,app}$. However, a recent study suggests that Lys pK_as are influenced less by carboxylates than calculations predict (*35*). Another important influence on ionization behavior is the local dielectric constant, a decrease in which could conceivably be the result of D58A conformational behavior and could subsequently raise the pK_a of an acidic group (*34*) and/or lower the pK_a of a Lys (*36*).

Contributions of Active-Site Ligands to Mg(II) Binding. As summarized in Table 2, the wild-type pH titration data were fit to a number of models in an effort to discern the number of critical ionizable groups and their respective pK_a values. For wild-type *Pvu*II endonuclease, models describing two equivalent and independent ionizable groups gave slightly better fits than those involving (weakly) cooperative

or nonequivalent ionizations. However, given the small differences in fit quality among these two-site models, one model cannot be advanced above the others. Thus, while it is possible that these ionizations interact and/or could be nonequivalent, these behaviors do not appear to distinct enough to warrant more complex models. Indeed, pK_as which differ by less than 0.7 pH units cannot be reliably resolved (*37*).

Both Mg(II) binding and pH studies are consistent with Glu68 contributing critically to Mg(II) binding. An attempt to identify the remaining ionizable Mg(II) ligand in the wild-type enzyme led to the observation of the unexpected behavior of D58A. Surprisingly, data describing the pH dependence of Mg(II) binding by this variant near physiological pH were best represented by a model involving three independent ionizations. The fact that there are multiple ionizable groups binding Mg(II) in D58A, a mutant enzyme in which one potential metal ion ligand is removed, indicates that groups in addition to Glu68 contribute to Mg(II) binding in this variant. *Pvu*II crystal structures were examined in order to identify potential metal ion ligands. Because the positions of residues 54 and 55 are not defined in the free *Pvu*II enzyme structure, it was necessary to use the coordinates in the DNA bound structure (*28*). Distances between carboxyl carbons (and the distal nitrogen in the case of Lys70) were measured; these atoms of Lys70, Asp58, Glu55, and Glu66 were all between 4 and 9 Å from Glu68. Except of course for Glu55, the same range is observed with the free enzyme. Considering that the enzyme changes conformation upon binding DNA, the side chains are flexible, and the D58A variant exhibits some conformational differences from wild-type (see below), Glu55, and/or Glu66 could be metal ion ligands in this variant. Since there is one ionization above pH 8 in this model, involvement of Lys70 is also a possibility. While there is no close match for the electrostatic environment of a restriction enzyme, pK_a values below 9 have been reported for a number of lysine residues (*36*, *38*−*40*).

Even though this study focuses on ionizable Mg(II) ligands, it is important to acknowledge that certainly not all Mg(II) ligands need be ionizable in the experimental region; most likely there are other contributors to metal ion affinity. Other crystallographically determined protein ligands for metal ions in restriction enzymes include Gln (*4*) and mainchain groups (*5*, *26*, *41*). Water-mediated contacts are very common (*12*) and also cannot be ruled out.

Correlation with Other Endonucleases. Of mechanistic interest is the correlation between the unusual pH dependence of Mg(II) binding by *Pvu*II endonuclease and the DNA binding behavior of *Mun*I endonuclease. For the latter type II enzyme, group(s) responsible (directly or indirectly) for sequence-specific DNA binding exhibited pK_a(s) somewhere between 6.5 and 8.3 (*42*). This was attributed to the abnormal ionization behavior of acidic residues responsible for metal ion binding. While substrate binding can indeed further influence active-site group pK_a values (*39*), the data are consistent with unusual ionization of conserved active-site residues in both enzymes. Interestingly, the behavior of the well-studied *Eco*RV endonuclease is distinct from this pattern. For this enzyme, the kinetically measured dissociation constant K_d^m for the catalytic metal ion [Mn(II) in this case] was constant between pH 6 and 8.5, indicating that the groups associated with metal ion binding are not ionizing in this range (*43*). It is intriguing that pK_a(s) for ionizing groups responsible for metal ion binding could significantly differ among restriction enzymes featuring the conserved acidic residue active-site motif. It is plausible this may reflect some mechanistic variations; further clarification via continued studies on a number of different restriction enzymes should prove informative.

Implications for Mg(II) Binding by a Low Activity Active-Site Variant. Given the central location of Asp58 and the consensus that mutations at active-site carboxyl groups abolish binding of required metal ions, our result that D58A binds Mg(II) is unexpected, but not necessarily inconsistent with a role in the support of catalysis. As mentioned above, determination of Mg(II)-binding stoichiometry for *Pvu*II endonuclease is not feasible via ^{25}Mg NMR spectroscopy. However, Hill analysis of wild-type Mg(II)-binding data yields a Hill coefficient (n_H) of 1.4 (Table 1), a value often interpreted as describing a weakly cooperative system (*44*, *45*) and consistent with at least two Mg(II) sites per *Pvu*II monomer active site. Indeed, a number of two-site models applied to these data produce fits with quality and K_as comparable to those reported in Table 1 (data not shown). Further, two sites per monomer active site for nonnative metal ions Ca(II) and Mn(II) were indeed observed by isothermal titration calorimetry (*12*).

In contrast, Hill analysis of Mg(II) binding by D58A produced an altered n_H value of 0.7 (Table 1). Below unity values are typically interpreted as negatively cooperative: occupancy of one site reduces the favorability of filling subsequent site(s) (*46*−*49*). This difference in Hill coefficient between wild-type and D58A is the simplest piece of evidence that Asp58 is important to metal ion binding in the active site. Further evidence emerges from the finding that multiple ionizable groups bind Mg(II) in D58A. Since two ionizable groups are involved in Mg(II) binding by the wild-type enzyme in the physiological pH range, it is apparent that adjustments are made in the active site to compensate for the loss of the Asp side chain. Upon mutation at residue 58, Mg(II) binding is preserved through the involvement of at least one ligand not critical to binding in the wild-type enzyme. The conformational configuration of the active site is likely affected in the process. This suggests that Asp58 is important to the proper placement or orientation of the Mg(II) ion(s) in the active site and would also explain how a variant which binds Mg(II) exhibits such low specific activity. How the presence of substrate affects such arrangements would require additional study (see above).

Conserved active-site carboxylate residues have been implicated as important to the structures of other hydrolytic enzymes (*50*, *51*). The results of our ^{19}F NMR spectroscopic studies of fluorinated wild-type and D58A *Pvu*II endonucleases suggest that Asp58 is important to enzyme conformation: No fewer than half of the 10 fluoro-Tyr residues experience changes in environment as a result of the D58A mutation, while only very modest chemical shift changes were apparent in E68A spectra (*12*). The unusual metal ion binding and ligand ionization behavior of D58A may indeed be attributable to distinct conformational behavior. Clearly more detailed structural studies would be necessary to explore this possibility for endonuclease active-site residues. In the

meantime, our studies serve as an indication that the contributions of conserved acidic residues to cofactor binding in nucleases can be more complex than generally considered.

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REFERENCES

1. Kulkosky, J., and Skalka, A. M. (1994) *Pharmacol. Ther. 61*, 185−203.
2. Belfort, M., and Roberts, R. J. (1997) *Nucleic Acids Res. 25*, 3379−3388.
3. Pingoud, A., and Jeltsch, A. (1997) *Eur. J. Biochem. 246*, 1−22.
4. Kostrewa, D., and Winkler, F. K. (1995) *Biochemistry 34*, 683−696.
5. Viadiu, H., and Aggarwal, A. K. (1998) *Nat. Struct. Biol. 5*, 910−916.
6. Duan, X. Q., Gimble, F. S., and Quiocho, F. A. (1997) *Cell 89*, 555−564.
7. Allingham, J. S., Pribil, P. A., and Haniford, D. B. (1999) *J. Mol. Biol. 289*, 1195−1206.
8. Sarnovsky, R. J., May, E. W., and Craig, N. L. (1996) *EMBO J. 15*, 6348−6361.
9. Gimble, F. S., and Stephens, B. W. (1995) *J. Biol. Chem. 270*, 5849−5856.
10. Nastri, H. G., Evans, P. D., Walker, I. H., and Riggs, P. D. (1997) *J. Biol. Chem. 272*, 25761−25767.
11. Huang, H.-W., and Cowan, J. A. (1994) *Eur. J. Biochem. 219*, 253−260.
12. José, T. J., Conlan, L. H., and Dupureur, C. M. (1999) *J. Biol. Inorg. Chem. 4*, 814−823.
13. Record, J., M. T., Lohman, T. M., and de Haseth, P. (1976) *J. Mol. Biol. 107*, 145−158.
14. Lindskog, S., and Nyman, P. O. (1964) *Biochim. Biophys. Acta 85*, 462−474.
15. Cowan, J. A. (1995) *The Biological Chemistry of Magnesium*, VCH, New York.
16. Neurohr, K. J., Drakenberg, T., and Forsen, S. (1983) *NMR Newly Accessible Nucl. 2*, 229−252.
17. Tsai, M.-D., Drakenberg, T., Thulin, E., and Forsen, S. (1987) *Biochemistry 26*, 3635−3643.
18. Reid, S. S., and Cowan, J. A. (1990) *Biochemistry 29*, 6025−6032.
19. Ehrlich, R. S., and Colman, R. F. (1995) *Biochim. Biophys. Acta 1246*, 135−141.
20. Lee, M. E., and Nowak, T. N. (1992) *Arch. Biochem. Biophys. 293*, 264−273.
21. Dupureur, C. M., and Hallman, L. M. (1999) *Eur. J. Biochem. 261*, 261−268.
22. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci. 4*, 2411−2423.
23. Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., and Schechter, A. N. (1972) *Biochemistry 11*, 541−547.
24. Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70−80.
25. Loizos, N., Silva, G. H., and Belfort, M. (1996) *J. Mol. Biol. 255*, 412−424.
26. Newman, M., Lunnen, K., Wilson, G., Greci, J., Schildkraut, I., and Phillips, S. E. V. (1998) *EMBO J. 17*, 5466−5476.
27. Jen-Jacobson, L., Engler, L. E., Lesser, D. R., Kurpiewski, M. R., Yee, C., and McVerry, B. (1996) *EMBO J. 15*, 2870−2882.
28. Cheng, X., Balendiran, K., Schildkraut, I., and Anderson, J. E. (1994) *EMBO J. 13*, 3927−3935.
29. Brenowitz, M., Jamison, E., Majumdar, A., and Adhya, S. (1990) *Biochemistry 29*, 3374−3383.
30. Engler, L. E., Welch, K. K., and Jen-Jacobson, L. (1997) *J. Mol. Biol. 269*, 82−101.
31. Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1967) in *The Enzymes* (Boyer, P. D., Ed.) Vol. VII, pp 665−868, Academic Press, New York.
32. Davoodi, J., Wakarchuk, W. W., Campbell, R. L., Carey, P. R., and Surewicz, W. K. (1995) *Eur. J. Biochem. 232*, 839−843.
33. Inoue, M., Yamada, H., Yasukochi, T., Kurochi, R., Miki, T., Horiuchi, T., and Imoto, T. (1992) *Biochemistry 31*, 5545−5553.
34. Antosiewicz, J., and McCammon, J. A. (1996) *Biochemistry 35*, 7819−7833.
35. Forsyth, W. R., Gilson, M. K., Antosiewicz, J., Jaren, O. R., and Robertson, A. D. (1998) *Biochemistry 37*, 8643−8652.
36. Stites, W. E., Gittis, A. G., Lattman, E. E., and Shortle, D. (1991) *J. Mol. Biol. 221*, 7−14.
37. Cleland, W. W. (1986) in *Investigation of Rates and Mechanisms of Reactions* (Bernasconi, C. F., Ed.) Vol. 6, Wiley, New York.
38. Zanetti, G. (1976) *Biochim. Biophys. Acta 445*, 14−24.
39. Jentoft, J. E., Gerken, T. A., Jentoft, N., and Dearborn, D. G. (1981) *J. Biol. Chem. 256*, 231−236.
40. Hartman, F. C., Milanez, S., and Lee, E. H. (1985) *J. Biol. Chem. 260*, 13968−13975.
41. Dudev, T., Cowan, J. C., and Lim, C. (1999) *J. Am. Chem. Soc. 121*, 7665−7673.
42. Lagunavicius, A., Grazulis, S., Balciunaite, E., Vainius, D., and Siksnys, V. (1997) *Biochemistry 36*, 11093−11099.
43. Stanford, N. P., Halford, S. E., and Baldwin, G. S. (1999) *J. Mol. Biol. 288*, 105−116.
44. Wyman, J., and Gill, S. J. (1990) *Binding and Linkage: Functional Chemistry of Biological Macromolecules*, University Science Books, Mill Valley.
45. Doan, L., Handa, B., Roberts, N. A., and Klumpp, K. (1999) *Biochemistry 38*, 5612−5619.
46. Huang, Y., Qureshi, I. A., and Chen, H. (1999) *Mol. Cell. Biochem. 197*, 195−201.
47. Taylor, A. M., Grobner, G., and Williamson, P. T. (1999) *Biochemistry 38*, 11172−11179.
48. Stache, R., Zeitler, R., and Reutter, W. (1997) *J. Biol. Chem. 272*, 24313−24318.
49. Neet, K. E., Keenan, R. P., and Tippett, P. S. (1990) *Biochemistry 29*, 770−777.
50. Dupureur, C. M., Li, Y., and Tsai, M.-D. (1992) *J. Am. Chem. Soc. 114*, 2748−2749.
51. Quirk, D. J., Park, C., Thompson, J. E., and Raines, R. T. (1998) *Biochemistry 37*, 17958−17964.
52. Petra, P. H., and Neurath, H. (1971) *Biochemistry 10*, 3171−3177.

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