The PD...(D/E)XK Motif in Restriction Enzymes: A Link between Function and Conformation†

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ABSTRACT: The active sites of Mg(II)-dependent nucleases feature a cluster of conserved charged residues which includes both acidic (Asp and Glu) and basic (Lys) side chains. In restriction enzymes, these side chains are part of the conserved PD...(D/E)XK functional sequence motif which has been implicated as being important in metal ion binding and catalytic steps. Recent work revealing the unusual behavior of the active site variant D58A of the representative *Pvu*II endonuclease prompted speculation that the array of charged groups in the nuclease active site may also be linked to conformational behavior [Dupureur, C. M., and Conlan, L. H. (2000) *Biochemistry 39,* 10921−10927]. To address this issue, we analyzed the conformational behavior of active site variants of *Pvu*II endonuclease using both NMR spectroscopic and thermodynamic methods. NMR spectroscopic analysis via ^{19}F and $^{1}H-^{15}N$ HSQC experiments indicates that a number of side chain and backbone amide groups are perturbed upon Ala substitution at conserved active site residues Asp58, Glu68, and Lys70. Spectral changes are particularly pronounced for the lowest-activity mutants (D58A and K70A). These changes are accompanied by perturbations in conformational stability. Ala substitution at each of these positions results in 2−5 kcal/mol of stabilization over the wild-type enzyme at pH 7.7, changes which constitute increases in $\Delta G_d^{H_2O}$ of 20−50%. The pH dependencies of mutant enzyme stabilities are distinct from those of the wild type, results which confirm that these ionizable groups strongly influence stability. Wild-type enzyme stability is correlated with the ionization of groups shown to be important to metal ion binding and orientation. Correlations between spectral changes and conformational stability indicate that the latter measurements may prove useful in the evaluation of site-directed mutant restriction enzymes. More importantly, these results indicate that structure−function relationships in restriction enzyme active sites can be complex, and that the ensemble of conserved charged residues which mediate DNA hydrolysis in Mg(II)-dependent nucleases constitutes a critical link between function and conformation.

The hydrolysis of phosphodiester bonds by nucleases is important to the synthesis, recombination, and degradation of nucleic acid sequences (*1*). The active sites of Mg(II)-dependent nucleases typically feature three acidic residues, all of which are highly conserved among enzymes featuring this activity (*2*−*4*). Among type II restriction enzymes, most of these side chains form the conserved PD...(D/E)XK functional sequence motif. Efforts to understand the mechanisms of these enzymes have focused on the structure−function relationships of these residues. Ala substitution of lysine and at least two of the conserved acidic residues typically results in dramatic losses in specific activity (*5*−*11*). In many nuclease systems, either crystallographic or solution studies have implicated at least two of the acidic residues as being important to Mg(II) binding (*5*, *10*, *12*−*18*). The role of the critical Lys residue appears to be less straightforward (*5*, *19*) but has been proposed to involve transition state stabilization (*20*).

As part of these efforts, we have been conducting structure−function studies of the representative *Pvu*II endonuclease, a restriction enzyme which utilizes Mg(II) to cleave DNA at the sequence 5′-CAG|CTG-3′ to produce blunt ends (*21*). As expected, this enzyme features the active site motif of Mg(II)-dependent nucleases. Crystallographic studies reveal the presence of conserved Glu55, Asp58, Glu68, and Lys70 in each active site (*22*). While the variant E55A retains near wild-type activity, Ala substitution at the latter three positions results in dramatic losses in specific activity (*10*). Direct Mg(II) binding studies indicate that Glu68 is critical to metal ion binding and implicate Asp58 as being important for proper positioning of this cofactor (*18*).

An important aspect of interpreting mutagenesis data and consequently understanding structure−function relationships is assessing the conformations of site-directed variants. A number of previous studies indicate that catalytic residues can impact the structure and stability of hydrolytic enzymes (*23*, *24*). Does the localization of so many charged residues, some of which could participate in repulsive interactions, come at some conformational cost? We have developed biosynthetic fluorination and ^{19}F NMR spectroscopy as a probe of *Pvu*II endonuclease conformation (*25*). The com-

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pact, 2 × 18 kDa homodimeric structure of *Pvu*II endonuclease makes it particularly amenable to such analyses. In our efforts to understand the role of Asp58 in the behavior of *Pvu*II endonuclease, we noted that fluorinated D58A exhibited ^{19}F NMR spectra distinct from that of the fluorinated wild-type enzyme, suggesting some conformational differences (*26*). In light of the very low specific activity and nearly wild-type Mg(II) binding behavior of this variant, this result prompted speculation that active site residues may be involved in conformational behavior. If indeed there is a link between the functional participation of conserved active site groups and conformation, then structure−function relationships among active site groups are correspondingly more complex. In this study, we address this question through the application of two-dimensional NMR methods and stability measurements to active site variants of the representative *Pvu*II endonuclease.

MATERIALS AND METHODS

Materials. ^{15}N-labeled ammonium chloride (>98%) was purchased from Isotec Inc. (Miamisburg, OH). Ultrapure guanidine hydrochloride was purchased from ICN Biomedicals (Aurora, OH).

Preparation of PvuII Endonucleases. The recombinant expression systems for WT,[1] E55A, D58A, E68A, and K70A *Pvu*II endonucleases in *Escherichia coli* (*27*) were kindly provided by P. Riggs of New England Biolabs. The cells were grown in minimal medium (M9 salts) supplemented with 10 μg/mL biotin, 10 μg/mL uracil, 50 μg/mL thiamine, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.5% glucose. Uniform incorporation of ^{15}N was accomplished by providing $^{15}NH_4Cl$ as the only nitrogen source, save 40 μg/mL Met required for growth. For 3-fluoro-Tyr incorporation, each amino acid except Tyr was added pure to a final concentration of 40 μg/mL. 3-Fluoro-Tyr was added to a final concentration of 1 mM at induction of expression with IPTG. Purification of all enzymes was accomplished using phosphocellulose chromatography and heparin−sepharose affinity chromatography as previously described (*25*). Proteins were concentrated using Amicon Centriprep and Centricon concentrators. All enzymes were quantitated using an ϵ_{280} of 36 900 M^{-1} cm^{-1}.

Activity Assays. Specific activities were measured using agarose gel electrophoresis of reaction products as described previously (*10*). Assays were conducted on 0.75 μg of DNA (120 nM sites) in NEB buffer 2 [10 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.9)] at 37 °C. Reactions were initiated with the addition of metal ion and quenched with EDTA. Reaction times were kept short (<60 min), with convenient rates modulated with enzyme concentration (0.1−10 nM dimer). Multiple rate determinations were averaged and errors analyzed using standard deviations.

NMR Analysis. NMR samples contained 350 or 500 μM *Pvu*II monomers in 50 mM Tris and 400 mM KCl (pH* 7.7) at 12 °C. These conditions minimize aggregation and maximize enzyme solubility and stability during experiments. ^{1}H and ^{19}F NMR spectra were collected on a Varian Unity Plus spectrometer at 500 and 470 MHz, respectively. Typically, ^{19}F NMR spectra were collected with 40 000 scans of 8192 points each in 10% D_2O unless otherwise indicated. ^{19}F chemical shifts are relative to external neat trifluoroacetic acid. HSQC spectra were acquired with 128 transients of 1024 points in the ^{1}H dimension and 160 points in the ^{15}N dimension. The ^{1}H spectral width was 7000 Hz, and the ^{15}N spectral width was 1600 Hz. ^{1}H chemical shifts are relative to DSS.

Determination of Conformational Stability ($\Delta G_d^{H_2O}$). Enzyme samples for GdnHCl denaturation were prepared from a 16 μM dimer stock solution of enzyme and a stock solution of GdnHCl near 8.5 M in 50 mM buffer and 100 mM KCl. To ensure optimum buffering capacities, EPPS buffer was used for measurements at pH 7.7, Bis-Tris-propane was used at pH 7.2 and 6.6, and MES was used at pH 5.5. The exact concentration of the GdnHCl stock was determined by the refractive index method (*28*). Both solutions, along with the buffer, were used to prepare 35−40 different solutions at various GdnHCl concentrations; the working concentration of the enzyme was 1 μM dimers in all cases.

Fluorescence emission intensities were collected on an SLM 8000 spectrofluorometer by using an excitation wavelength of 295 nm (2 nm band-pass) and recording the emission intensity at 330 nm (4 nm slit width). The temperature was maintained with a thermostated compartment at 25 °C. At least four intensity measurements were averaged at each point. Data were applied to a two-state folding model for homodimers as previously described (*29*).

RESULTS

NMR Spectroscopic Analysis of the Active Site Variant Conformation. To determine how active site groups affect conformation, we applied to Ala variants two complementary spectroscopic methods: one-dimensional ^{19}F NMR spectroscopy, which is sensitive to environmental changes of ^{19}F nuclei incorporated into Tyr side chains (*25*, *30*, *31*), and two-dimensional ^{1}H−^{15}N HSQC experiments, which probe the backbone of the enzyme (*32*).

(1) ^{19}F NMR Spectroscopy. Our development of ^{19}F NMR spectroscopy as a routine method for evaluating restriction enzyme conformation has been described previously (*25*). Since the locations of Tyr residues are established by X-ray crystallography (*22*), it is possible to discern the extent of conformational changes with this method. Studies are performed on a *Pvu*II endonuclease in which ≈70% of the Tyr residues are replaced with 3-fluorotyrosine. 3-Fluorotyrosine *Pvu*II endonuclease has sequence specificity and specific activity indistinguishable from those of the native enzyme (*25*). Each *Pvu*II endonuclease subunit has 10 Tyr residues, three of which lie in or near the active site: Tyr94, which is 3 Å away from the Glu68 carboxylate in the apoenzyme crystal structure (*33*); Tyr124, which is 8 Å away from the Glu68 carboxylate group; and Tyr67, which is adjacent to Glu68 in this structure but points away from the active site, placing the aromatic ring more than 10 Å away from either carboxylate group. The remaining Tyr residues are distributed throughout the remainder of the enzyme and, upon substitution with 3-fluorotyrosine, serve as probes for other parts of the enzyme structure.

[1] Abbreviations: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FPLC, fast protein liquid chromatography; FY, 3-fluorotyrosine; GdnHCl, guanidine hydrochloride; HSQC, heteronuclear single-quantum correlation; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

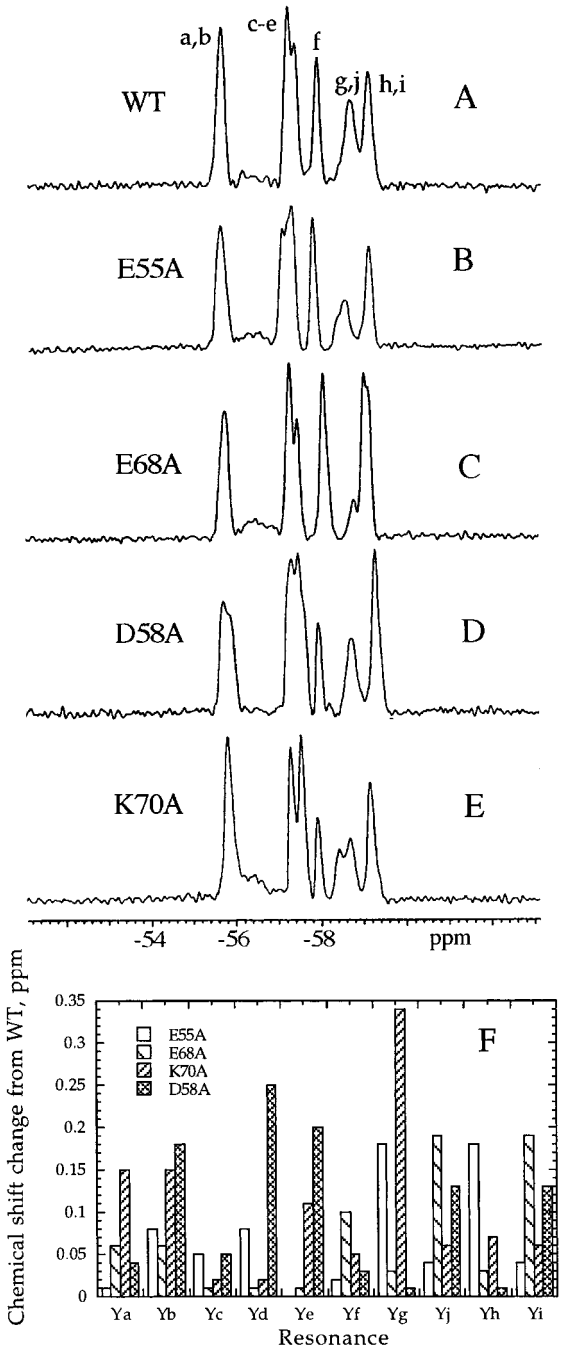

FIGURE 1: One-dimensional ^{19}F NMR spectra (470 MHz) of 3-fluorotyrosine *Pvu*II endonucleases: (A) WT, (B) E55A, (C) E68A, (D) D58A, and (E) K70A. The conditions were as follows: 0.5 mM monomers, 50 mM Tris, and 400 mM KCl (pH* 7.7) in 10% D_2O at 12 °C. Chemical shifts are relative to TFA. (F) Bar graph summarizing chemical shift differences from the wild-type spectrum.

Panels A−E of Figure 1 feature the ^{19}F NMR spectra of FY-WT, FY-D58A, FY-E68A, FY-E55A, and FY-K70A. A number of changes are clearly evident among the mutant spectra and were quantitated using chemical shift measurements. For these 3-fluorotyrosine *Pvu*II endonucleases, these measurements are reproducible from sample to sample to within 0.02 ppm.

The correlation of shifted resonances in fluorinated variant spectra with those of the fluorinated wild-type enzyme is the result of a cumulative process involving a number of samples under various conditions which give rise to perturbations and diminished degeneracies. For example, unique chemical shifts for Ya and Yb are revealed in the presence of Ca(II) (*25*). The same is true of Yc−e, for which three resonances are distinguished upon Ca(II) binding. When shifts of overlapping resonances are observed relative to FY-WT, the most conservative distinctions are made. For example, if a chemical shift is observed downfield of Yc−e, it is assigned to resonance Yc. If the movement is upfield, it is assigned to Ye. If a peak moves out between two clusters, it is assigned to the closest group, unless there are no other chemical shift changes evident there. Rarely do multiple resonances shift by so much that the correlation with a control chemical shift is ambiguous. In practice, assignments constitute the most conservative changes in chemical shift. Indeed, the possibility that some resonances could be shifting by far more than noted cannot be ruled out.

The movements of resonances relative to those of the fluorinated wild-type enzyme are catalogued in Table 1. Changes of ≥ 0.1 ppm offer insight into differences among the spectra (Figure 1F). As expected, the spectrum of FY-E55A is most like that of FY-WT, with only Yg and Yh shifting significantly. For FY-E68A, Yf, Yi, and Yj shift, with all other resonances remaining unperturbed. These spectra provide contrast for those of FY-K70A and FY-D58A, for which four and five resonances shift at least 0.1 ppm, respectively. Because there are fewer than four 3-fluorotyrosines near the active site, the number and magnitude of these shifts in FY-D58A are consistent with longer-range perturbations relative to FY-WT.

(2) $^{1}H-^{15}N$ HSQC NMR Spectroscopy. Figure 2 features the HSQC spectra of wild-type *Pvu*II endonuclease and active site variants. Ideally, approximately 170 cross-peaks are expected to result from backbone and side chain amide groups of the enzyme. However, since *Pvu*II endonuclease is a large protein by NMR standards (2×18 kDa), it is reasonable that about 60% of the resonances are visible under these experimental conditions; the remainder likely correspond to spectral overlap and resonances with poor signal-to-noise ratios due to unfavorable exchange and/or relaxation behavior. Even with this fraction of visible peaks, such HSQC spectra provide valuable information about how mutations affect backbone conformational behavior. As illustrated, the backbone conformations of variant *Pvu*II endonucleases are similar to that of the native enzyme (black resonances are overlapping), indicating that the overall fold of the protein is preserved in all variants. The overlay of the FY-E55A spectrum with wild-type spectra illustrates how similar spectra can be. This provides clear contrast for the remaining low-activity variants. For E68A, D58A, and K70A, nonoverlapping intensities (red and green) are readily visible and reproducible. This is indicative of some perturbations to the protein backbone as a result of the mutations.

Conformational Stability. While NMR spectroscopy provides detailed structural information, it cannot assess the importance of observed spectral changes to enzyme conformation. Changes in protein conformational stability are very sensitive to the importance of side chains and their respective interactions (*34*). These experiments are also attractive because a variety of conditions can be explored with a minimum of material. Indeed, if a correlation between spectral changes and changes in conformational stability can be established, these latter measurements could prove useful

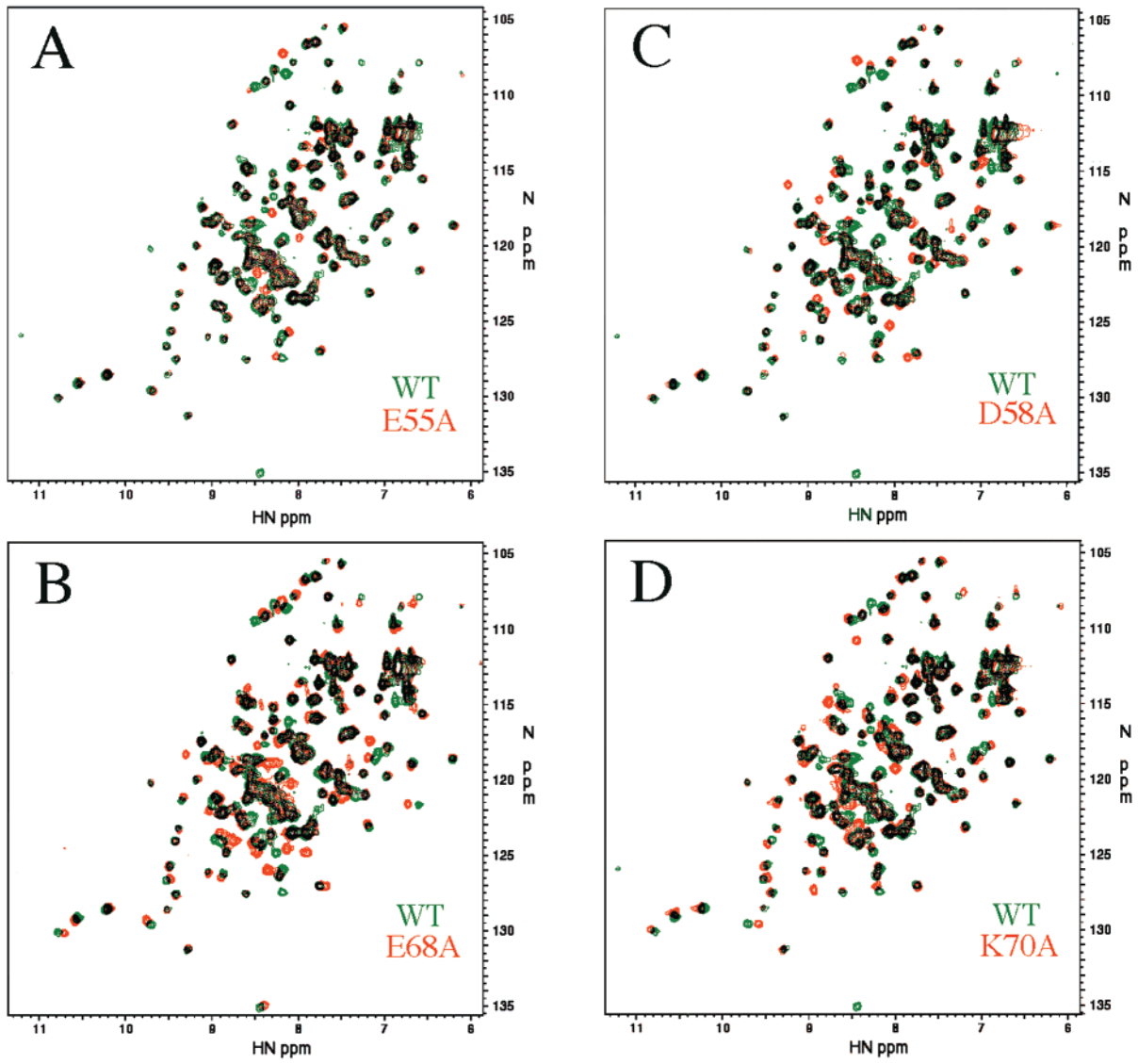

FIGURE 2: Overlaid $^{1}H-^{15}N$ HSQC spectrum of wild-type *Pvu*II endonuclease overlaid with spectra of the (A) E55A, (B) E68A, (C) D58A, and (D) K70A mutants. Wild-type resonances are green, mutant resonances red, and overlapping intensities black. The sample conditions were as follows: 350 μM *Pvu*II monomers, 50 mM Tris, and 400 mM KCl (pH* 7.7) in a 90% H_2O/10% D_2O mixture at 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

in those instances where NMR spectroscopic analysis is not feasible.

Measurements of conformational stability are most conveniently achieved through titration of a chemical denaturant and observation of accompanying spectral changes. Because circular dichroism spectroscopy is specifically sensitive to secondary structure (*35*), it is ideal for observing unfolding of proteins. However, *Pvu*II endonuclease produces a very weak circular dichroism spectrum ($[\theta]_{222} = -12$ deg cm^2 dmol^{-1}), necessitating the use of another method. *Pvu*II endonuclease has four Trp residues, two of which are significantly protected from solvent (*22*). In the presence of 8 M GdnHCl, intrinsic Trp fluorescence is quenched by approximately 50%, making this method suitably sensitive to *Pvu*II endonuclease unfolding. As shown in Figure 3, the data for the wild type and all variants appear to be consistent with a two-state unfolding model and were analyzed with the standard equation

$$\Delta G_d = \Delta G_d^{H_2O} - m[\text{GdnHCl}] \quad (1)$$

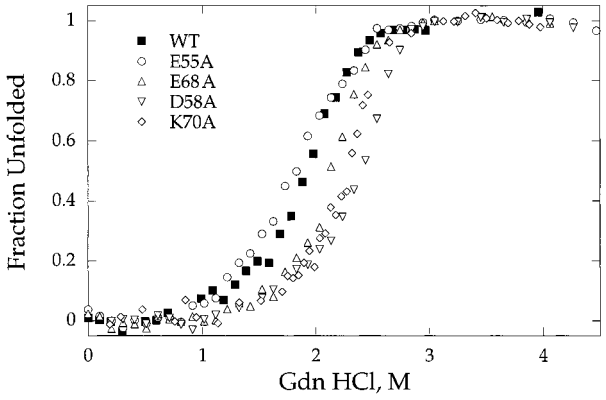

FIGURE 3: Guanidine HCl-induced denaturation curves for wild-type and mutant *Pvu*II endonucleases. The identity of each curve is as given in the inset. The conditions were as follows: 50 mM EPPS and 100 mM KCl (pH 7.7) at 25 °C.

where ΔG_d is the Gibbs free energy change at various concentrations of GdnHCl, $\Delta G_d^{H_2O}$ is that at zero GdnHCl, and m is a constant related to the susceptibility of the enzyme

Table 1: Summary of ^{19}F Chemical Shifts for Wild-Type and Mutant 3-Fluorotyrosine *Pvu*II Endonucleases[a]

	activity[b]	Ya	Yb	Yc	Yd	Ye	Yf	Yg	Yj	Yh	Yi
WT	1	−55.72	−55.72	−57.31	−57.31	−57.47	−57.99	−58.78	−58.78	−59.24	−59.24
E55A	10^{-1} [c]	−55.73	−55.80	−57.26	−57.39	−57.47	−57.97	−58.60	−58.60	−59.28	−59.28
E68A	10^{-2}	−55.78	−55.78	−57.30	−57.30	−57.48	−58.09	−58.81	−58.81	−59.05	−59.05
K70A	10^{-4}	−55.87	−55.87	−57.33	−57.33	−57.58	−57.94	−58.44	−58.71	−59.18	−59.18
D58A	10^{-3}	−55.76	−55.90	−57.36	−57.56	−57.67	−58.02	−58.79	−58.79	−59.37	−59.37

[a] Chemical shifts are in parts per million relative to external trifluoroacetic acid. Mutant enzyme resonances which differ from those of the wild-type fluorinated enzyme (FY-WT) by at least 0.1 ppm are underlined. FY-WT, FY-D58A, and FY-E68A chemical shift data were adapted from ref *26*. [b] Relative specific activity. [c] From ref *10*.

toward denaturation by the denaturant. Near the assay pH (7.7), Ala substitution at Glu55 results in a modest decrease in stability. In contrast, removal of either carboxylate at Asp58 or Glu68 by separately mutating the side chain to Ala (i.e., D58A and E68A) results in an increase in stability of about 2−3 kcal/mol (Table 2). Interestingly, the mutant K70A, in which the amino group of Lys is replaced with Ala, is a remarkable 5 kcal/mol more stable than the wild type. These $\Delta\Delta G_d^{H_2O}$ values represent substantial increases (20−50%) in overall conformational stability.

pH Dependence of Stability. Since all of the active site groups in question are ionizable, some measure of their contributions to stability can be estimated by measuring conformational stability at various pH values. Previous studies have attributed unusual p$K_{a,app}$ values (centered around 6.7) to active site groups contributing to Mg(II) binding (*18*). If it is the ionization behaviors of these side chains that are responsible for reduced enzyme stability, then the wild-type enzyme will be more stable at pH values at which these groups are protonated. As shown in Table 2 and Figure 4, wild-type *Pvu*II endonuclease is approximately 2 kcal/mol more stable at pH 5.5 than at pH 7.7, consistent with the protonation of destabilizing ionizing groups. To determine the influence of Glu55, Asp58, Glu68, and Lys70 on the pH dependence of stability, $\Delta G_d^{H_2O}$ values for E55A, D58A, E68A, and K70A were also determined at pH 5.5. In all cases, stability was preserved. The stability of E55A exhibits a pH dependence similar to that of the wild type; i.e., it is more stable at lower pH values. The remaining mutants were about as stable at pH 5.5 as at pH 7.7, consistent with the involvement of these side chains in the pH dependence of enzyme stability.

To better understand the dependence of stability on pH, stabilities of these enzymes were also obtained at intermediate pH values (7.2, 6.6, and 6.1). For WT, the $\Delta G_d^{H_2O}$ at pH 6.6 is almost precisely intermediate between 5.5 and 7.7, indicating a transition throughout this pH range. The high stabilities of E55A at pH 6.6 and 7.2 are consistent with a shift in the p$K_{a,app}$ of this stability dependence toward higher pH. As summarized in Table 2 and Figure 4, the pH dependencies of the stabilities of the low-activity variants K70A, D58A, and E68A are more complex, with $\Delta G_d^{H_2O}$ being optimal at intermediate pH (≈6.5).

DISCUSSION

Influence of Active Site Groups on Stability. It is becoming clear that the arrangement of charged groups in restriction enzyme active sites can result in unusual and complex behavior. In a study designed to test the hypothesis that Glu68 and Asp58 are Mg(II) ligands, we noted that ionizable groups responsible for metal ion binding behavior exhibited a collective p$K_{a,app}$ of 6.7 (*18*), a value considered unusual for acidic residues. This study reveals a link among the ionization behavior of active site residues, conformation, and stability. The pH dependence of wild-type *Pvu*II endonuclease conformational stability correlates well with the unusual pK_a values of active site residues known to be involved in Mg(II) binding (Glu68, among others). The enzyme is more stable at pH 5.5 where the ionizing Mg(II) ligands are protonated than at pH 7.7, where they are deprotonated. The fact that the pH dependencies of mutant enzyme stabilities are different than those of the wild type confirms that these ionizable groups strongly influence wild-type stability. Active site mutations D58A, E68A, and K70A unmask the influence of both stabilizing and destabilizing deprotonation events. Additional experiments would be necessary to definitively identify which ionizable groups are involved (*36*), but it appears likely that one or more of them are active site groups. All groups, both stabilizing and destabilizing, are present in WT, which may be why this variability is masked in the wild-type enzyme.

In early theoretical work, Wyman predicted that acidic groups with unusual pK_a values are destabilizing to proteins (*37*). In addition to *Pvu*II endonuclease, there are a number of examples which support this theory. Of particular interest here are those cases which involve active site acidic groups. The most studied case is that of Glu35 of lysozyme, which exhibits an unusual pK_a of 6.1; substitution of this residue with Ala results in protein stabilization of 1.8 kcal/mol (*38*). Similarly, Glu172 in the active site of xylanase exhibits an unusual pK_a (6.8) and destabilizes the enzyme at pH values above this value (*39*). Another related example is the buried conserved Asp26 of thioredoxin, which has a pK_a well above 7 (*40*). This enzyme is 2 kcal/mol more stable at neutral pH than above it; when Asp26 is replaced with Ala, the stability increases by 4.6 kcal/mol and appears to be pH-independent in the ionizing range (*40*). Finally, RNase HI features a cluster of conserved acidic residues both in and near the active site, some of which are implicated in metal ion binding. One of the remaining residues, Asp134, has a more normal pK_a and is important for activity, but destabilizes the enzyme by 2 kcal/mol (*41*).

The increased stability of these active site variants begs the question of why the side chains of these groups are destabilizing. Understanding the role of electrostatics in protein stability is receiving increased attention. Unless involved in salt bridge interactions (*42*), acidic groups are often destabilizing (*36*). A number of previous workers have attributed the destabilizing effect of active site acidic groups to the unfavorable proximity of another acidic group. The

Table 2: Summary of Wild-Type and Mutant *Pvu*II Endonuclease Conformational Stabilities[a]

pH	WT			D58A			E68A			E55A			K70A		
	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_d^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	m (kcal mol^{-1} M^{-1})
7.7	6.7 ± 0.2	1.90	−3.71	10.0 ± 0.4	2.34	−4.43	8.5 ± 0.1	2.07	−4.29	5.3 ± 0.3	1.82	−3.13	11.7 ± 0.3	2.29	−5.29
7.2	–	–	–	–	–	–	–	–	–	7.3 ± 0.1	1.89	−4.05	13.5 ± 0.2	2.25	−6.16
6.6	7.6 ± 0.2	1.93	−4.13	13.0 ± 1.1	2.36	−5.66	13.3 ± 0.6	2.14	−6.39	9.2 ± 0.1	1.94	−4.95	17.1 ± 0.2	2.29	−7.62
6.1	–	–	–	–	–	–	–	–	–	–	–	–	13.5 ± 0.3	2.30	−6.01
5.5	8.6 ± 0.1	1.82	−4.93	10.8 ± 0.2	2.11	−5.27	8.2 ± 0.4	1.80	−4.77	9.3 ± 0.4	1.80	−5.39	12.6 ± 0.2	2.11	−6.16

[a] $D_{1/2}$ is the concentration of denaturant at the midpoint of the unfolding transition. Definitions of $\Delta G_d^{H_2O}$ and m are as given in the text.

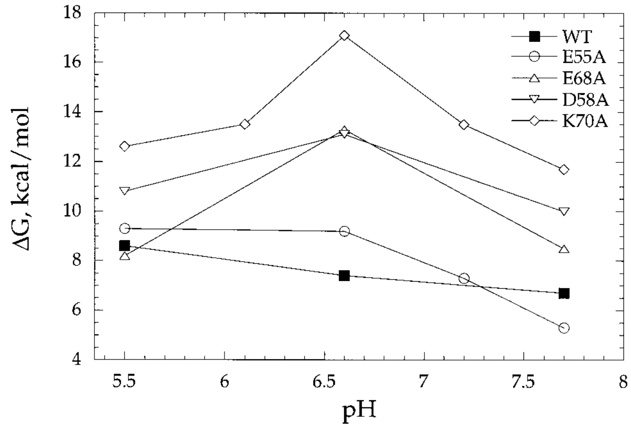

FIGURE 4: pH dependence of $\Delta G_d^{H_2O}$ for wild-type and mutant *Pvu*II endonucleases. The identity of each curve is as given in the inset. The conditions were as follows: 1 μM dimers in 50 mM buffer (see Materials and Methods) and 100 mM NaCl at 25 °C.

destabilizing effect of Glu35 of lysozyme has been attributed to interactions with Asp52 (*38*). Similarly, a destabilizing interaction between Glu172 and Glu78 in xylanase has been identified (*39*). Given the proximity of conserved Asp58, Glu68, and Glu55, such an effect could well be operative for *Pvu*II endonuclease and thus may be a general characteristic of Mg(II)-dependent nucleases. However, the destabilizing effect of Lys70, a positive charge amidst a number of negative charges, cannot be rationalized in this fashion.

If the destabilizing effects are predominantly repulsive, then perhaps Mg(II) binding could relieve some of these interactions in the active site. To address this issue, we measured the conformational stability of wild-type *Pvu*II endonuclease in the presence of Mg(II). Interestingly, there was only a modest change in stability relative to the others we reported [5.0 vs 6.7 kcal/mol in the absence of Mg(II)]. At first, this result seemed puzzling; however, two results from the laboratory lend some perspective and provide possible explanations. First of all, Mg(II) binds *Pvu*II endonuclease with a weak but characteristic affinity for nucleases ($K_d \approx 1$ mM) (*18*). This means that such binding is likely disrupted very early in the titration with GdnHCl, which gives it little opportunity to influence the unfolding and folding processes. In those cases where metal ions stabilize proteins, they bind with greater affinities (*43*−*45*). Second, Mg(II) binding induces very little structural perturbation (see below). Thus, it is reasonable that the influence of Mg(II) on stability would be limited.

A final possible explanation for the behavior described above is that despite some reports which attribute destabilization to repulsive electrostatic interactions (*38*, *39*), this may not be the dominant effect among Mg(II)-dependent nuclease active sites. First of all, the interactions in this active site cannot be fairly characterized as entirely repulsive. There are both positive (Lys and often multiple divalent cations) and negatively charged (multiple Glu and Asp residues) moieties in proximity, resulting in opportunities for both favorable and unfavorable interactions. This may explain why the overall effect appears to be more simple than it is. Gaining increased attention are the magnitude of desolvation effects and their influence on unusual ionization behavior and destabilization. When the enzyme folds, favorable interactions between acidic groups and solvent are sacrificed

to form the active site (*46*). In this less solvated environment, charged acidic groups are destabilizing and elevated pK_a values are favored (*36*). This effect may explain why a repulsive interaction model is not entirely satisfactory. Whatever the rationalization, the interplay between functional groups and stability is clearly complex, and the fact that the mutant pH profiles of stability are distinct from that of the wild type is consistent with the important involvement of active site groups in pH-dependent stability.

Correlation between Stability and NMR Conformational Analysis. In this study, we couple measurements of conformational stability with two forms of NMR spectroscopic analysis. The first method involves biosynthetic incorporation of 3-fluorotyrosine incorporation and ^{19}F NMR spectroscopy. Developed as the first solution probe of restriction enzyme conformation, it has been useful in detecting metal ion-dependent conformational changes in *Pvu*II endonuclease (*25*). Here it is applied to characterize the conformational behavior of active site variants.

While differential effects of fluorination on the active site variants cannot be ruled out, the experiments described here reveal that all of the fluorinated mutant enzymes exhibit some chemical shift perturbation. Some of this is likely due to the change in the environment of fluorotyrosines in proximity to the active site. For example, we have previously noted that titration of the paramagnetic Mn(II) ion resulted in the selective broadening of the Yg,j resonances (*25*). However, in the spectra of FY-K70A and FY-D58A presented here, approximately half (four to five) of the resonances are shifted by more than 0.1 ppm. Because there are fewer than four 3-fluorotyrosines near the active site, the number and magnitude of these shifts are consistent with longer-range perturbations. Backbone perturbations were also probed using ^{1}H$-^{15}$N HSQC spectroscopy. Overlay spectra reveal that except for E55A, there are multiple perturbations of backbone amide protons in the mutant enzymes. This reflects our continuing observation that these probes report the magnitudes of conformational changes in a comparable fashion (C. M. Dupureur, unpublished results).

To put these spectral changes in perspective, consider that Mg(II) binding, which has been confirmed using ^{25}Mg NMR spectroscopy (*18*), causes little perceptible change in either ^{19}F (*25*) or HSQC spectra (data not shown). This is despite the fact that there are a number of 3-fluorotyrosine side chains in proximity to the active site. In light of these results and the dramatic changes in conformational stability, the spectral perturbations seen for the active site variants appear to be indicative of accompanying conformational adjustments. This effect appears to be most dramatic for K70A and D58A, which exhibit the lowest specific activities of the active site variants (10^{-4} and 10^{-3} less than the wild-type value, respectively). Since the null activity of K70A can be rationalized in terms of a critical loss in transition state stabilization (*20*), it is unclear how much of the kinetic perturbation can be attributed to the conformational changes detected here. However, interpretation of D58A behavior is more straightforward. This variant binds Mg(II) with wild-type affinity, but with some perturbation of cooperativity and ligand identities (*18*). When these data are coupled with the spectral changes and increased stability relative to that of the wild type, it becomes clear that mutation at Asp58 results in conformational differences that impair function, implicating Asp58 as being linked to the maintenance of an active enzyme conformation.

Measurements of changes in conformational stability often reflect structural changes that can be detected by NMR spectroscopy (*23*, *47*). When stability has been compromised, significant spectral changes are not unexpected. Our results indicate that even where mutation results in stabilization, there is a positive correlation between large changes in $\Delta G_d^{H_2O}$ and conformational adjustments as observed by NMR spectroscopy. This suggests that measurements of conformational stability might be a useful substitute for more detailed but less feasible conformational analysis by NMR spectroscopy.

Link between Function and Conformation. The most attractive structure−function relationships are certainly those in which side chains contribute to a single behavior. However, in a growing number of examples, protein structure has proven to be more subtle, linking multiple behaviors through a number of key residues. Residues established as critical to catalysis of enzymes have been shown to be involved in protein stability and conformation. In a number of cases, it appears that stability has been sacrificed for the sake of function. Mutation of the catalytic His274 and Asp375 in pig citrate synthase resulted not only in profound losses in specific activity but also in dramatic increases in thermal stability (*48*). In the α-subunit of tryptophan synthase from *Salmonella*, catalytically inactive active site variants (including E49F and D60A) exhibit greater thermal stabilities (up to 3 kcal/mol) than the wild type (*49*). Similar results were obtained for active site variants of staph nuclease; NMR spectroscopic data confirm a conformational response to mutation of the catalytic Glu43 (*47*). Mutations of the charged active site residues of barnase known to interact with the RNA substrate (Lys27, Arg59, and His102) gave expected large reductions in catalytic efficiency through K_m effects, but also resulted in increases in stability relative to the wild-type enzyme (*50*). Finally, substitution of the functionally important acidic residues in the well-studied lysozyme (at Glu11 and Asp20) increased stability by 0.7−1.7 kcal/mol (*51*).

In cases in which catalytic residues are linked to conformational behavior, it is not always true that active site residues *destabilize* enzymes. Sometimes the effect is the opposite. Catalytic residues not only mediate activity but also are critical to the conformational integrity of the enzyme. Removal of catalytic residue Asp99 from the His-Asp catalytic diad of phospholipase A2 results not only in inactivation but also in the loss of half (4.7 kcal/mol) of the conformational stability of the enzyme; further, the Ala variant has a molten globule-like structure (*23*). Although less dramatic, active site variants of RNase A exhibit similar behavior (*24*).

Among all of these examples, the effects of mutating active site residues on *Pvu*II endonuclease conformation and stability stand out as some of the most dramatic (increases up to 5 kcal/mol, or 50% of $\Delta G_d^{H_2O}$) and may be indicative of the conformational cost of forming the active sites of Mg(II)-dependent endonucleases. The results reported here lend strong support to the notion that instead of functional active site side chains being appendages to the structural context of an enzyme, in some cases they can be better described as integral to structure, constituting critical links between function and conformation.

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