NMR Studies of Restriction Enzyme−DNA Interactions: Role of Conformation in Sequence Specificity†

Cynthia M. Dupureur*

Department of Chemistry and Biochemistry, University of Missouri−St. Louis, St. Louis, Missouri 63121

Received December 14, 2004; Revised Manuscript Received February 1, 2005

ABSTRACT: Sequence specific DNA binding proteins are thought to adopt distinct conformations when binding to target (cognate) and nontarget (noncognate) sequences. There is both biochemical and crystallographic evidence that this behavior is important in mediating sequence recognition by the Mg(II)-dependent type II restriction enzymes. Despite this, there are few systematic comparisons of the structural behavior of these enzymes in various complexes. Here, $^{1}H-^{15}N$ HSQC NMR spectroscopy is applied to *Pvu*II endonuclease (2 × 18 kDa) in an effort to better understand the relationship between sequence recognition and enzyme conformational behavior. Spectra of the free enzyme collected in the absence and presence of metal ions indicate that while there is a modest backbone conformational response upon binding Ca(II), this does not occur with Mg(II). Substrate binding itself is accompanied by very dramatic spectral changes consistent with a large-scale conformational response. HSQC spectra of the enzyme bound to cognate (specific) and noncognate (nonspecific) oligonucleotides in the presence of Ca(II) are dramatically distinct, revealing for the first time the structural uniqueness of a *Pvu*II cognate complex in solution. The strong correlation between NMR spectral overlap and crystallographic data (C_α rmsd) permits characterization of the nonspecific *Pvu*II complex as being more similar to the free enzyme than to the specific complex. Collectively, these data support the notion that it is the DNA, not the metal ion, which promotes a unique conformational response by the enzyme. It therefore follows that the principle role of metal ions in complex formation is one of driving substrate affinity and stability rather than conformationally priming the enzyme for substrate binding and sequence recognition. These results not only provide valuable insights into the mechanism of protein−DNA interactions but also demonstrate the utility of NMR spectroscopy in structure−function studies of these representative nucleic acid systems.

Sequence discrimination by nucleic acid binding proteins is one of the most remarkable examples of molecular recognition in biochemistry. Target sequences of 6 bp typically occur once every few thousand base pairs, yet they are efficiently recognized by restriction enzymes (*1*). While their recognition sites are typically larger, transcriptional regulators perform this same feat (*2*). To find this proverbial "needle in a haystack", proteins must have evolved an integrated set of mechanisms for accomplishing this task. An attractive model is that of facilitated diffusion, in which proteins bind nucleic acids nonspecifically and then proceed along the helix until the target site is located (*3*).

In addition to favorable diffusion kinetics, this model has an important structural component: To facilitate rapid motion along the helix, interactions between the protein and nontarget sequences must be less intimate than that with the target sequence (*4*, *5*). The motivation to better understand the structural basis of sequence discrimination has led to an abundance of crystallographic studies of proteins complexed to their target sequences. These structures reveal a rich library of interactions at the atomic level (*6*). Among these are structures of the type II restriction enzymes, the Mg(II)-dependent homodimeric DNA hydrolases that have revolutionized molecular biology (*1*). Thermodynamic studies indicate that sequence discrimination among restriction enzymes is accomplished with differential affinities for cognate (specific) versus noncognate (nonspecific) sequences (*7*, *8*). Early evidence for a relationship between sequence specificity and conformational behavior emerged from biochemical studies of *Eco*RI and *Eco*RV endonucleases (see the Discussion) (*7*, *9*, *10*). As is the case for many specific DNA binding proteins, most direct information about conformational behavior has been obtained from crystallographic studies of specific DNA complexes (*1*, *11*). The structures of more than dozen restriction enzymes have been reported to date.

To best understand the magnitudes of protein conformational changes associated with DNA binding, cognate complex structures must be compared to the appropriate reference structures, more specifically, those of free protein and nonspecific complexes. Despite this imperative, there is only a small number of systems for which this information has been published (*12*−*14*). One impediment to studying reference structures is the necessity of determining multiple structures to extract conformational information. In this respect, NMR spectroscopy is an attractive complementary technique. Using the convenient and highly sensitive $^{1}H-^{15}N$ HSQC[1] experiment (*15*), the conformational consequences of a wide variety of interactions can be assessed in

† This work was supported by NIH Grant GM67596.

* To whom correspondence should be addressed. Telephone: (314) 516-4392. Fax: (314) 516-5342. E-mail: cdup@umsl.edu.

[1] Abbreviations: HSQC, heteronuclear single-quantum coherence; *pH, pH uncorrected for the presence of deuterium; rmsd, root-mean-square deviation; TROSY, transverse relaxation optimized spectroscopy.

10.1021/bi0473758 CCC: $30.25
Published on Web 03/09/2005

solution in a matter of hours. In addition, because it is a solution technique, NMR spectroscopy can provide information about population distributions, exchange, and molecular motion (*16*, *17*).

We have been pursuing restriction enzyme structure−function relationships in *Pvu*II endonuclease, which recognizes the 5′-CAG|CTG-3′ sequence and in the presence of Mg(II) cleaves at the central base step to achieve blunt ends (*18*). Like many restriction enzymes, *Pvu*II endonuclease also utilizes metal ions to achieve effective sequence discrimination. In the presence of 10 mM Ca(II), a cofactor which does not promote cleavage, the cognate complex stability is 5 kcal/mol greater than that observed in the absence of added metal ions (*19*). While the thermodynamic contribution of this cofactor to sequence specificity is clear, the role of these ions in structural contributions to this process could be better understood. While there are structures of the cognate *Pvu*II complex and the free enzyme (*20*, *21*), no structural information about nonspecific *Pvu*II complexes has been reported.

As the smallest well-characterized restriction enzyme (156 amino acids, 18 × 2 kDa), *Pvu*II endonuclease is an ideal system for exploring the power of NMR spectroscopic studies of restriction enzymes. To that end, biosynthetic fluorination and ^{19}F NMR spectroscopy were applied to examine *Pvu*II conformational changes associated with the binding of divalent cations (*22*). More recently, uniform isotopic labeling and ^{1}H−^{15}N HSQC spectroscopy were applied to assess conformational effects of active site mutations (*23*). In this study, NMR spectroscopy is applied for the first time to examine the conformational basis of restriction enzyme sequence discrimination. More specifically, this work addresses the following questions. Do divalent metal ions conformationally prepare the enzyme for DNA binding? How conformationally distinct are cognate and noncognate *Pvu*II complexes in solution? In addition to examining these important issues, we also use the data collected here to evaluate the enzyme for the application of more sophisticated techniques. As will become clear, even in the absence of resonance assignments, these spectra provide an unprecedented level of detail about restriction enzyme solution conformational behavior.

EXPERIMENTAL PROCEDURES

Materials. Chelex resin was purchased from Bio-Rad (Hercules, CA). Puratronic $MgCl_2$ and $CaCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). Concentrations of stock solutions were determined by flame atomic absorption spectroscopy using a Perkin-Elmer AAnalyst 700 spectrophotometer. All buffers were applied to a Chelex column to remove adventitious metal ions. Subsequent pH adjustments were made with metal-free nitric acid. All solutions were determined by atomic absorption spectroscopy to be metal-free to the limits of detection (*24*).

Preparation of PvuII Endonucleases. The recombinant expression system *Pvu*II endonuclease in *Escherichia coli* (*25*) was kindly provided by P. Riggs of New England Biolabs. The cells were grown in minimal media (M9 salts) supplemented with 10 μg/mL biotin, 10 μg/mL uracil, 50 μg/mL thiamine, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.5% glucose. Uniform incorporation of ^{15}N was accomplished by providing $^{15}NH_4Cl$ as the only nitrogen source, save 40 μg/mL Met required for growth. Purification of all enzymes was accomplished using phosphocellulose chromatography and heparin−Sepharose affinity chromatography as previously described (*22*). Proteins were concentrated using Amicon Centriprep and Centricon concentrators, and adventitious metal ions were removed via exhaustive dialysis against metal-free buffer (*26*). In repeated determinations, we have been unable to detect metal ions in enzyme samples prepared in this fashion. Thus, the enzyme is termed "metal-free" to the limits of detection. All apoenzymes were quantitated using an ϵ_{280} of 36 900 M^{-1} cm^{-1} for the monomer subunit and handled with metal-free sterile pipet tips and sterile plasticware to prevent contamination.

Preparation of Oligonucleotides. The oligonucleotide 5′-TGACCAGCTGGTC-3′ (self-complementary 13mer) was purchased desalted from the Biopolymer Synthesis Facility at the California Institute of Technology (Pasadena, CA) and purified by reverse phase and ion exchange HPLC. The self-complementary 14mer strand 5′-CGACCAGCTGGTCG-3′ containing a 3′-amidate linkage at the scissile phosphate was purchased HPLC purified from Transgenomic (Boulder, CO). DNA was quantitated using ϵ_{260} values provided by the vendor. All oligonucleotide concentrations are expressed with respect to the duplex. Using Centricons, DNAs were rendered metal-free through at least two exchanges of >90% volume with deionized distilled water. Subsequent handling was accomplished with metal-free pipet tips and sterile plasticware. Samples were stored in sterile water at 4 °C for immediate use or lyophilized for storage.

NMR Analysis. NMR samples contained 650 μM *Pvu*II monomers in 25 mM Tris and 200 mM KCl at pH* 7.7 and 25 °C. DNA and metal ion concentrations were as indicated. ^{1}H−^{15}N TROSY HSQC NMR spectra were collected on a 600 MHz Varian Inova spectrometer located at Washington University. HSQC spectra were acquired with 128 transients of 1024 points in the ^{1}H dimension and 100 points in the ^{15}N dimension. The ^{1}H spectral width was 8000 Hz, and the ^{15}N spectral width was 1800 Hz. ^{1}H chemical shifts are relative to DSS. Data were processed with resolution enhancement and sine-bell shifting.

Overlap Analysis. Spectra were plotted using thresholds that permitted the best visualization of peaks without including a lot of noise. In overlays, comparable thresholds were used for both source spectra. The intensities for source spectra were summed (NIH Image 1.63), and any difference was used to generate and apply a correction factor. The fraction of shifted area in overlaid spectra was obtained by dividing this intensity (either red or blue) by the area represented in the reference spectrum (the sum of black and opposing color intensities). Calculating overlap with both red and blue intensities generated results that varied up to several percent. For this reason, only differences of >5−10% were interpreted.

C_α root-mean-square deviations (rmsds) between all common C_α atoms in entire crystal structures were calculated using SwissPDB Viewer 3.7 software. PDB entry 4RVE was edited to contain only two subunits arranged as a homodimer. For the 2BAM, 3BAM, and 1RVB structures, only the subunit containing the metal ions was used in the calculation. Since the *Bam*HI structures can only be compared as monomers, rmsds for both monomer and dimer forms for

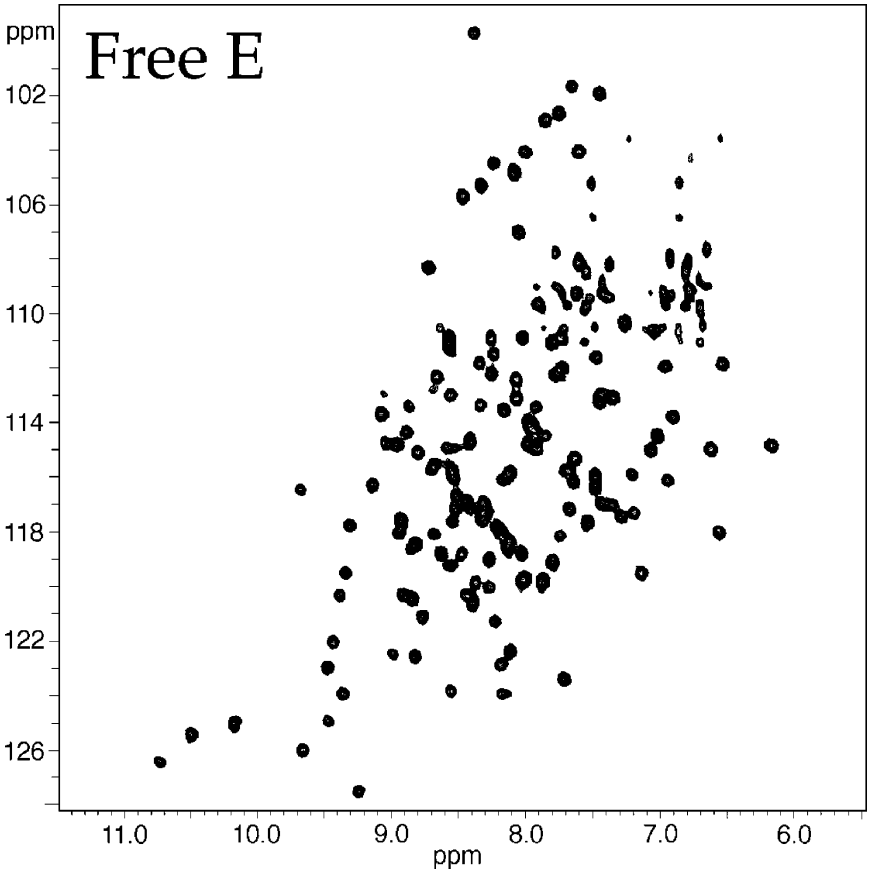

FIGURE 1: $^{1}H-^{15}N$ TROSY HSQC spectrum of *Pvu*II endonuclease at 600 MHz. Conditions: 0.65 mM enzyme monomers, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, and 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

all structures involved in relevant comparisons are also included.

RESULTS

Sample Conditions. Since this is the first comprehensive NMR study of restriction enzyme−DNA interactions, and part of the goal of the study is to evaluate *Pvu*II endonuclease for more detailed studies, some attention to sample conditions is warranted. Ideal protein NMR samples are high in concentration, very low in ionic strength, yet stable to many hours of data collection at room temperature. Given that restriction enzymes are more stable in high salt and have a tendency to aggregate at high concentrations (*22*, *27*), these constraints present a special challenge. In previous calorimetric studies requiring high concentrations of enzyme (1 mM monomers), we noted that *Pvu*II endonuclease is quite stable at room temperature at ionic strengths around 400 mM (*28*). While we have collected a number of spectra under these conditions (*22*, *23*), it can be difficult to tune some probes and sensitivity to the coils is compromised. Therefore, a series of trial HSQC spectra were collected and compared at 50 mM Tris and 400 mM NaCl, 25 mM Tris and 200 mM NaCl, and 12 mM Tris and 100 NaCl, all at pH 7.7, a pH close to optimum for activity. At the lowest ionic strength, the enzyme precipitated after long periods of room-temperature acquisition. Spectra collected between 0.5 and 1 mM *Pvu*II monomers at 25 mM Tris and 200 mM NaCl gave high-quality spectra with a minimum of sample degradation. For most samples prepared in this fashion, a good signal can be obtained with 32 transients without the application of TROSY. However, samples containing phosphoramidate oligonucleotide were of reduced quality and benefited from additional transients and TROSY. To facilitate spectral comparisons, all spectra presented here were collected in this fashion.

Free Enzyme Spectra. The first restriction enzyme NMR studies involved biosynthetic fluorination and ^{19}F NMR spectroscopy (*22*). In those studies, 10 Tyr residues served as side chain probes of local protein environments. $^{1}H-^{15}N$ HSQC spectroscopy provides a different perspective: Since these experiments correlate amide protons to their corresponding nitrogens, these experiments report on backbone environments throughout the protein structure, providing a "fingerprint" spectrum of the enzyme (*29*). Figure 1 features the $^{1}H-^{15}N$ HSQC spectrum of free *Pvu*II endonuclease. *Pvu*II endonuclease has eight Pro residues, nine Asn residues, and six Gln residues. Ideally, 180 cross-peaks are expected from backbone and side chain amide groups of the enzyme in this region. The application of a 600 MHz field, resolution enhancement, and the TROSY modification (*30*) have resulted in an improvement in sensitivity compared to that of HSQC spectra published previously (*23*). The limits of signal-to-noise ratios, the number of discernible peaks in this spectrum is essentially equal to that expected. This spectral quality indicates a potential for more detailed studies.

Metal Ion-Mediated Enzyme Conformational Changes. An important issue in restriction enzyme structure−function studies is the role of metal ions in DNA sequence recognition

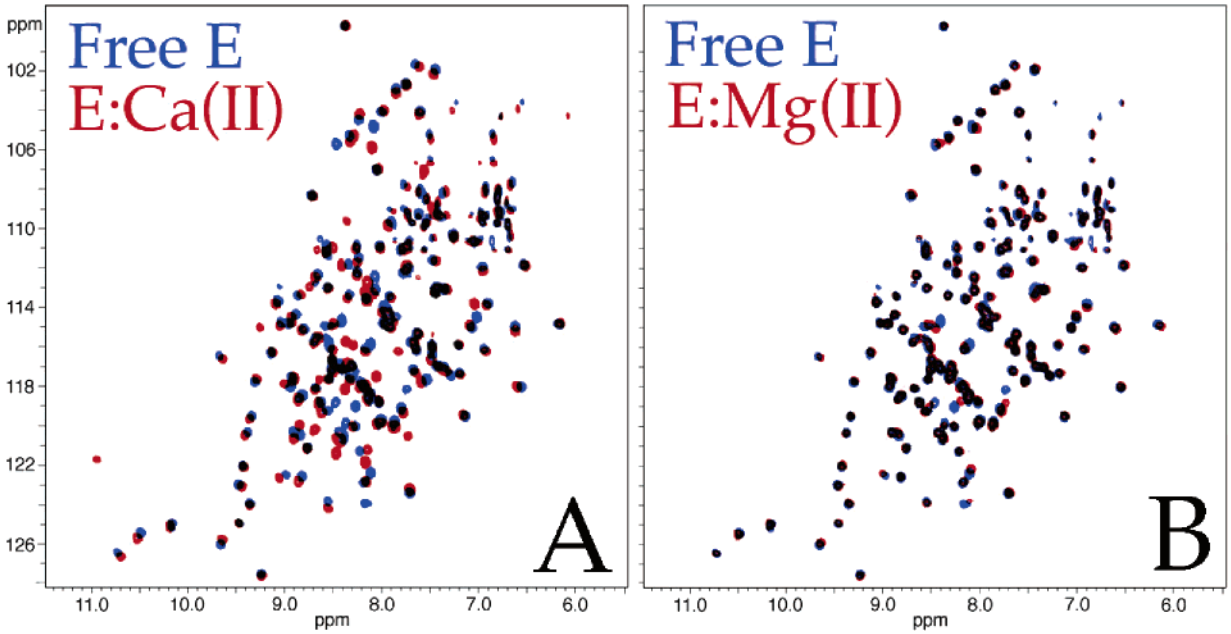

FIGURE 2: Conformational effects of metal ion binding. $^{1}H-^{15}N$ TROSY HSQC spectra of *Pvu*II endonuclease in the presence of (A) Ca(II) (red) and (B) Mg(II) (red) at 600 MHz. Spectra are overlaid with the free enzyme spectrum (blue) for easy comparison. Conditions: 0.65 mM enzyme monomers, 10 mM metal chloride salt, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, and 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

and cleavage. This question naturally extends to conformational behavior. Specifically, do metal ions promote enzyme conformational changes in preparation for DNA complex formation? A few early structure−function studies suggested that metal ions might be involved in conformational changes associated with DNA binding and cleavage (see the Discussion) (*9*, *31*). More recent studies of metal ion−enzyme interactions have been helpful in providing structural and thermodynamic perspectives. Both Ca(II) and Mg(II) bind *Pvu*II endonuclease with low millimolar dissociation constants (*28*, *32*). While ^{25}Mg NMR spectroscopy demonstrated that the latter ion binds the enzyme (*32*), this event was could not be detected by calorimetry or ^{19}F NMR spectroscopy. Recently, the structure of *Pvu*II endonuclease bound to Mg(II) was determined (*33*); remarkably, the C_α rmsd between this structure and that of the free enzyme is only 0.67 Å. Collectively, these data suggest that Mg(II) does not affect the conformation of the enzyme, and is therefore not likely involved in conformational priming. Binding of Ca(II), on the other hand, is associated with obvious enthalpic and ^{19}F spectral signatures consistent with a conformational response (*22*, *28*). How significant is this response in the protein backbone, and how does it compare to conformational changes associated with DNA binding? The behaviors of enzyme−metal complexes provide a valuable opportunity to gauge the sensitivity of HSQC spectra to divalent metal ion binding. They also serve as calibrations of pattern changes for spectral studies of enzyme−substrate complexes.

To determine the extent of backbone conformational adjustments upon the addition of common *Pvu*II cofactors, spectra were collected in the presence of 10 mM Ca(II) and in the presence of 10 mM Mg(II). Panels A and B of Figure 2 compare the spectrum of the free enzyme (blue) with that obtained in the presence of Ca(II) versus Mg(II) (red). There is a clear change in the cross-peak pattern in the presence of Ca(II). There are both substantial overlap and evidence that the environments of a number of backbone amide protons have been perturbed.

As described below, sequence specific assignments will be a significant undertaking. However, in the meantime, a measure of the degree of conformational response to metal ion binding can be assessed with spectral overlays. While one cannot directly correlate the magnitudes of chemical shift changes with structural changes, the extent to which spectra display overlapping intensities indicates a provisional measure of conformational similarities. To facilitate comparisons, the intensity which is common to both spectra is presented in black. Cross-peak intensities that are unique to one spectrum are either blue or red as indicated. In addition to the overall visual impression this coding provides, varying degrees of overlap can be quantitated. Using imaging software, peak areas (as opposed to volumes) were normalized, integrated, and compared. The fraction of overlapping area is obtained by dividing the black intensity by the area represented in the reference spectrum (the sum of black and either blue or red intensity as appropriate). Using this simple analysis, approximately 60% of the apoenzyme cross-peak area overlaps with the spectrum obtained in the presence of Ca(II).

As shown in Figure 2B, a much more modest conformational response is observed upon addition of Mg(II). Only a few backbone amide protons have shifted; the amount of black intensity indicates a high degree of overlap with the apoenzyme spectrum. Indeed, more than 80% of the intensity is unaffected by the addition of Mg(II).

DNA Complexes. While the spectra described above are informative, the conformational changes of greatest interest are those associated with DNA binding and sequence discrimination. The stabilities of these complexes are critical to spectral quality. Our extensive kinetic and equilibrium DNA binding studies indicate that Ca(II)-promoted binding of cognate DNA is avid. At 10 mM $CaCl_2$, the equilibrium

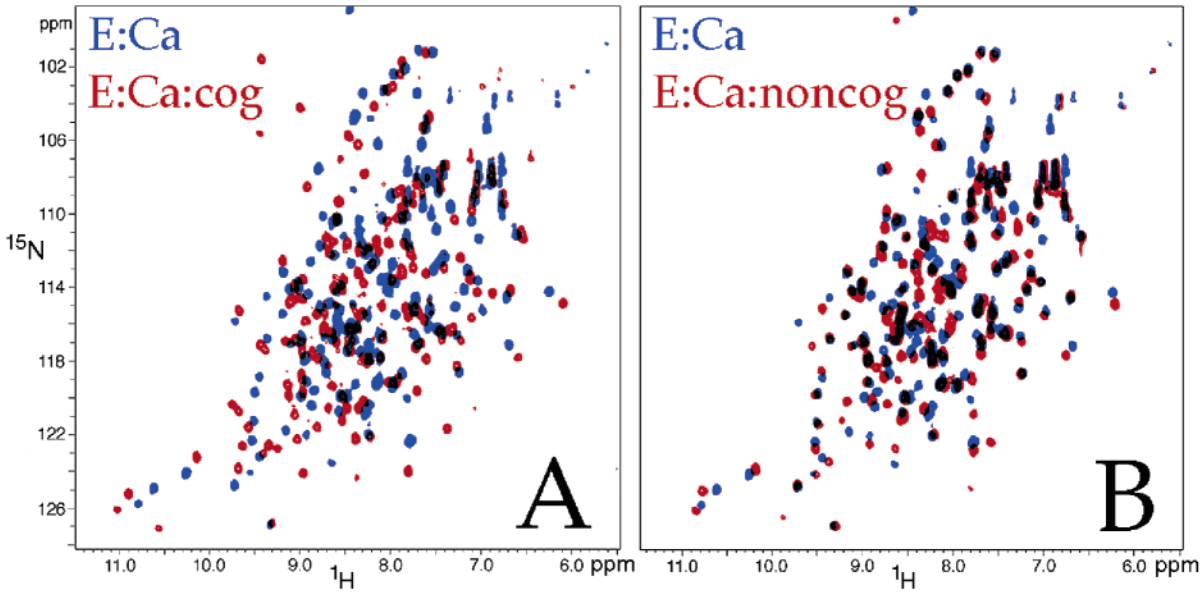

FIGURE 3: Conformational effects of DNA binding. (A) Overlay of ^{1}H−^{15}N TROSY HSQC spectra of the Ca(II)−*Pvu*II endonuclease complex (blue) and upon the addition of 1 equiv of self-complementary 13mer cognate duplex DNA (red) at 600 MHz. (B) Overlay of ^{1}H−^{15}N TROSY HSQC spectra of a Ca(II)−*Pvu*II endonuclease complex (blue) and upon the addition of 1 equiv of self-complementary 13mer noncognate DNA (red). Conditions: 0.65 mM enzyme monomers, 10 mM $CaCl_2$, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, and 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

constant for this process is 50 pM at an ionic strength of ~100 mM. Moreover, dissociation rates are very slow, 10^{-3} s^{-1} (*34*). Thus, exchange broadening and concomitant increases in line width should not affect spectral quality.

To evaluate the magnitude of enzyme conformational changes upon cognate DNA binding, a spectrum of an enzyme−Ca(II)−DNA complex was collected and overlaid with that of an enzyme−Ca(II) complex (Figure 3A). The quality of the spectrum obtained in the presence of DNA is quite high. Indeed, increased chemical shift dispersion and some improvement in line width are observed. We speculate that substrate binding alleviates some aggregation behavior. The most dramatic aspect of the overlay is that there is very little intensity common to both spectra (~20%). This is consistent with a very large scale conformational change upon addition of DNA. The magnitude of the difference between the enzyme−Ca(II) and enzyme−Ca(II)−DNA spectra is consistent with a model in which the DNA, rather than the metal ions, dominates the conformational response of the enzyme to substrate binding.

Specific versus Nonspecific DNA Complexes. The role of protein conformational behavior in sequence recognition is of particular interest not only to students of restriction enzymes but also to those of nucleic acid binding proteins in general (*14*). Currently, there are only two endonuclease systems for which there are crystal structures of the free enzyme and both cognate (specific) and noncognate (nonspecific) DNAs (*Eco*RV and *Bam*HI) (*12*, *13*). In both systems, there are clear conformational differences between these structures (*1*).

To examine this question for the first time with a restriction enzyme in solution, the spectrum of *Pvu*II endonuclease was collected in the presence of a noncognate oligonucleotide and Ca(II) (E:Ca:noncognate, red). Figure 3B is an overlay of this spectrum with that of the enzyme−Ca(II) complex (blue). While there are obvious differences, particularly between 9 and 10 ppm in the ^{1}H dimension, the degree of overlap is clearly higher than for the enzyme−Ca(II)−cognate DNA complex (50 vs 20% for the specific complex). This indicates that the enzyme conformational response to binding nonspecific DNA is much less dramatic than it is upon binding cognate DNA.

It is therefore not a surprise that there are very dramatic differences between the enzyme−Ca(II)−cognate (red) spectrum and the enzyme−Ca(II)−noncognate (blue) spectrum. This is best appreciated in Figure 4. While the overall patterns are sufficiently similar to confirm the identity of the protein, many nuclear environments are different. Approximately 80% of the intensity of each spectrum is distinct from that of the opposing spectrum. The scale of these differences indicates that the conformation of the enzyme in each complex is quite distinct.

Conformational Effect of Divalent Metal Ion Cofactors in DNA Complexes. The large conformational changes induced by DNA and the small changes induced by metal ions indicate that DNA is the principal effector of enzyme conformational behavior. These solution data support the observations of crystallographers, who note that in crystalline form, restriction enzyme conformation is predominantly driven by DNA binding (see the Discussion). It should also follow that spectra of enzyme−DNA complexes supported by different ions or in the absence of metal ions should exhibit substantial similarities.

A familiar complication in testing this hypothesis is the ability of Mg(II) to support cleavage in native substrates. The search for suitable nonhydrolyzable substrate analogues with which to conduct restriction enzyme−DNA studies in the presence of Mg(II) has been longstanding. This was our motivation for exploring the behavior of *Pvu*II endonuclease toward phosphoramidate (PN) oligonucleotides, DNAs in which the 3′-oxygen of the scissile phosphate is replaced with nitrogen (*35*). In this recent study, we reported that a

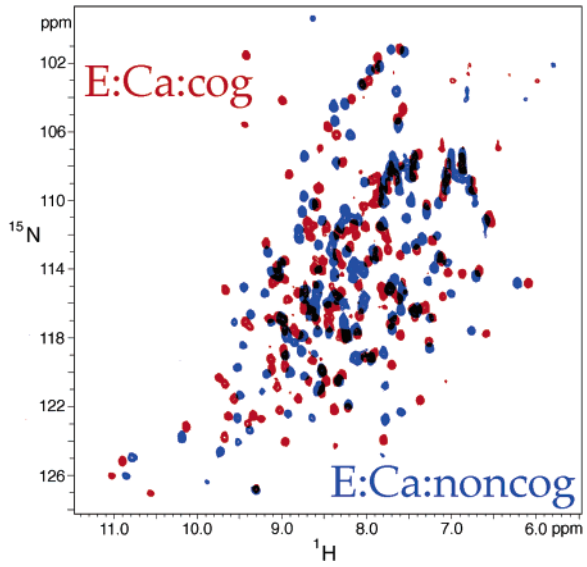

FIGURE 4: Specific vs nonspecific complexes. ^{1}H−^{15}N HSQC spectra of *Pvu*II endonuclease complexed to cognate (red) and noncognate (blue) self-complementary 13mer duplexes in the presence of Ca(II) at 600 MHz. Conditions: 0.65 mM enzyme monomers, 0.65 13mer strands, 10 mM $CaCl_2$, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, and 25 °C. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

self-complementary 14mer containing a PN linkage at the scissile position is stable in the presence of *Pvu*II endonuclease and Mg(II). Further, in the presence of Ca(II), the enzyme binds cognate PN with the same affinity as it does the corresponding native cognate duplex (K_d = 300 pM at 10 mM $CaCl_2$). Further, as highlighted in overlay spectra in Figure 5A, ^{1}H−^{15}N HSQC peak patterns are quite similar for the enzyme bound to PN and native DNA complexes in the presence of Ca(II). The overlap intensity is ~60%; closer inspection of the peak patterns reveals that most of the peaks shift only slightly. In most cases, one can easily assign corresponding pairs of red and blue peaks. These data suggest that while there a substantial number of small chemical shift differences, at least on a global scale, enzyme conformation when bound to native or PN duplexes is comparable, providing some confidence that PN mimics the native substrate in familiar ways.

Of course, the value of phosphoramidates is the ability to conduct experiments on enzyme−PN complexes in the presence of Mg(II). At 10 mM $MgCl_2$, the K_d for cognate PN binding is ~10 nM; the K_d obtained in the absence of added metal ions is within experimental error of this value. This indicates that metal ion-independent binding is considerably more avid for cognate PN than we observe for the corresponding native duplex. Such properties facilitate the formation of enzyme−PN complexes under a variety of conditions, making phosphoramidates suitable substrate analogues for spectroscopic studies.

The simplest way to test the hypothesis described above is to compare spectra of enzyme−Mg(II)−PN and enzyme−PN complexes, that is, to a complex formed in the absence of added divalent cations. Since the enzyme binds Mg(II) with little to no conformational response, one does not have to account for this event when interpreting the ternary complex spectra. Second, the high affinity of the enzyme for PN in the absence of metal ions makes the formation of the complex under NMR conditions (i.e., high ionic strength) facile. With this modified oligonucleotide, it is indeed possible to saturate the enzyme under these conditions, yielding the blue spectrum in Figure 5B (Enzyme:PN). Obvious differences between this spectrum and that of the free enzyme (Figure 1) indicate a DNA complex has indeed been formed. For example, the constellation of peaks between 9 and 10 ppm ^{1}H at 102−106 ppm ^{15}N are unique to cognate complexes. Comparison with the spectra of the same complex obtained after the addition of Mg(II) (red) reveals strikingly obvious similarities and a high degree of overlap (85%). Thus, while we cannot completely rule out that PN adversely affects Mg(II) binding [i.e., that Mg(II) may not be part of the enzyme−PN complex], these data support the general observation that DNA dominates the enzyme conformational response, and that divalent metal ions do not conformationally prime the enzyme for substrate binding.

A more complex comparison is that of DNA complexes formed in the presence of Ca(II) and Mg(II). To that end, the spectrum of the enzyme−Ca(II)−PN complex (blue) is overlaid with that of the enzyme−Mg(II)−PN complex (red) (Figure 5C). Upon initial examination, it appears that there is not a large degree of overlap between these spectra (15%). Visual examination of the spectral overlap indicates that while there are clear chemical shift differences, the overall peak patterns are obviously comparable. This is particularly clear in the top left and bottom left and right of the spectrum, where individual peak movement is easier to appreciate. If one mentally corrects for the magnitude of the conformational changes induced by Ca(II) alone (spectral overlaps would not be instructive here), it would appear that the differences are more dramatic than would be accounted for by considering the modest effect of Ca(II) binding on enzyme conformation. A rationale for these differences is considered below.

DISCUSSION

Conformational Behavior of Restriction Enzymes. Conformational behavior is an important means of mediating the function of nucleic acid enzymes (*36*, *37*). Because of their exquisite sequence selectivity and metal ion dependence, restriction enzymes serve as valuable model systems for examining this phenomenon. Biochemical data provided the first evidence that DNA recognition, binding, and cleavage by restriction enzymes were coupled to conformational behavior. Conformational differences between specific and nonspecific DNA binding by *Eco*RI endonuclease emerged from footprinting analyses (*38*−*40*). Changes in ethylation interference patterns also suggested that the enzyme adapts to various DNA structures during binding events (*38*). Additional evidence for this behavior stemmed from fluorescence studies of DNA binding and cleavage of *Eco*RI and *Eco*RV endonucleases (*7*, *9*).

Metal Ion and DNA Binding. The role of metal ions in DNA sequence recognition and cleavage continues to be an important focus of restriction enzyme structure−function studies. One of the first indications that metal ions might be involved in conformational priming emerged from the aforementioned *Eco*RV cleavage assays conducted using tryptophan fluorescence spectroscopy. The authors attributed intensity differences to *Eco*RV conformational changes

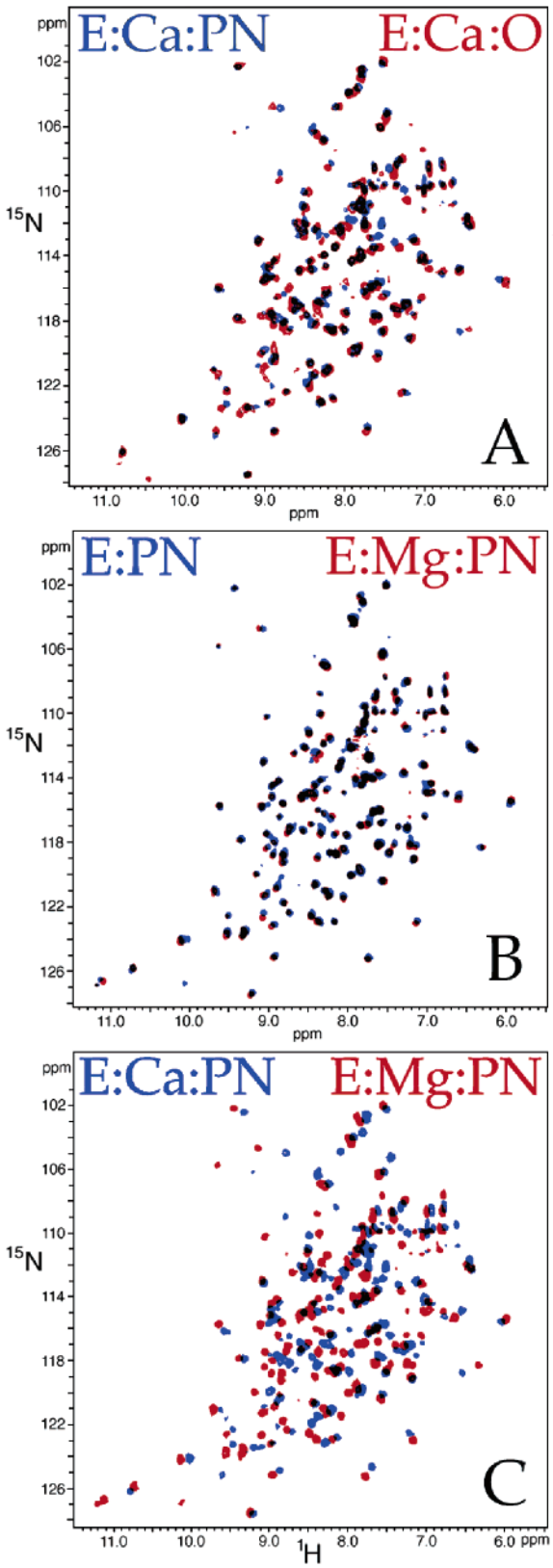

FIGURE 5: DNA complexes in the presence of Ca(II) and Mg(II). (A) Overlay of ^{1}H−^{15}N TROSY HSQC spectra of *Pvu*II endonuclease complexed to native (O, red) and phosphoramidate-containing (PN, blue) 14mer cognate duplexes in the presence of 10 mM Ca(II). (B) Overlay of ^{1}H−^{15}N TROSY HSQC spectra of *Pvu*II endonuclease complexed to a phosphoramidate-containing 14mer cognate duplex in the absence (blue) and presence of Mg(II) (red). (C) Overlay of ^{1}H−^{15}N TROSY HSQC spectra of *Pvu*II endonuclease complexed phosphoramidate-containing 14mer cognate duplexes in the presence of Ca(II) (blue) and Mg(II) (red) at 600 MHz. Conditions: 0.65 mM enzyme monomers, 0.65 14mer strands, 25 mM Tris, 200 mM KCl, 10% D_2O, pH* 7.7, and 25 °C. Where appropriate, the metal chloride concentration is 10 mM. FIDs of 1024 points from 128 scans were processed with line broadening and a sine-bell shift. Chemical shifts are relative to DSS.

associated with the binding of Mg(II) equivalents (*9*). There is also evidence that these effects might differ among restriction enzymes: While soaking of *Eco*RI−DNA cocrystals with Mg(II) leads to in situ cleavage (*41*), conformational changes associated with cleavage were not permitted by an *Eco*RV cocrystal [PDB entry 1RVB (*42*)]. While it has long been established that the *Eco*RI enzyme does not require metal ions to form high-affinity complexes (*27*), more recent studies indicate that metal ions drive substrate affinity for a number of other restriction enzymes (*19*, *43*, *44*). The data presented here indicate that there is a modest backbone conformational response upon binding Ca(II), supporting previous evidence that there is little conformational response to this event (*22*, *28*).

The functional relevance of these results emerges from NMR spectra of enzyme−DNA complexes presented here. Crystallographic studies indicate that regardless of the presence or identity of divalent cations, enzyme conformations in DNA complexes are comparable, making DNA the principal effector of enzyme conformational behavior. C_α rmsds between restriction enzyme−DNA structures with metals and those without them are generally small. Seven structures containing either Mg(II), Mn(II), or Ca(II) differ from metal-independent structures by less than 1 Å (Figure 6) (*12*, *13*, *42*, *45*−*49*). If one accepts that phosphoramidates and native oligonucleotides interact similarly with metals and enzyme, then the signature peak patterns of a DNA-bound enzyme in the absence of added metal ions and the high degree of spectral overlap with that observed in the presence of Mg(II) would support this view. This would relegate metal ions as instruments of driving DNA *affinity*, rather than large scale conformational behavior. This is an important insight into the mechanism of specific protein−DNA interactions.

What remains to be reconciled, however, are the spectral differences between the spectra of the enzyme−Ca(II)−PN and enzyme−Mg(II)−PN complexes. One possibility is that the PN substitution differentially affects Ca(II) and Mg(II) in the context of an enzyme complex. Strictly speaking, we cannot rule this out. Since observing native complexes in the presence of Mg(II) will always be technically difficult, we may never know if such differential behavior would exist in unmodified substrate complexes. We attributed the stability of PN in the presence of the enzyme and Mg(II) to local conformational effects (*35*); these could be more obvious in the presence of Ca(II) than Mg(II), possibly due to the increased ionic radius of the former ion (0.99 and 0.65 Å, respectively). However, the extensive nature of the spectral differences does suggest something more widespread than an effect at one location. Another consideration is the exquisite sensitivity of the chemical shift parameter. A series of small differences in nuclear environments could cause small chemical shift changes in a significant number of resonances without dramatic differences in the overall peak pattern or conformation. This is supported by the obvious similarities in the peak patterns; it is easy to see many pairs of red and blue peaks in all sectors of the spectrum that differ in chemical shift only slightly. This behavior is far more extensive and obvious in Figure 5C than in any of the other overlays. In the latter cases, many peaks either do not move much at all (Figure 2) or move so dramatically (Figure 4) very few can be confidently correlated. Such observations are consistent with the remaining data, i.e., that some

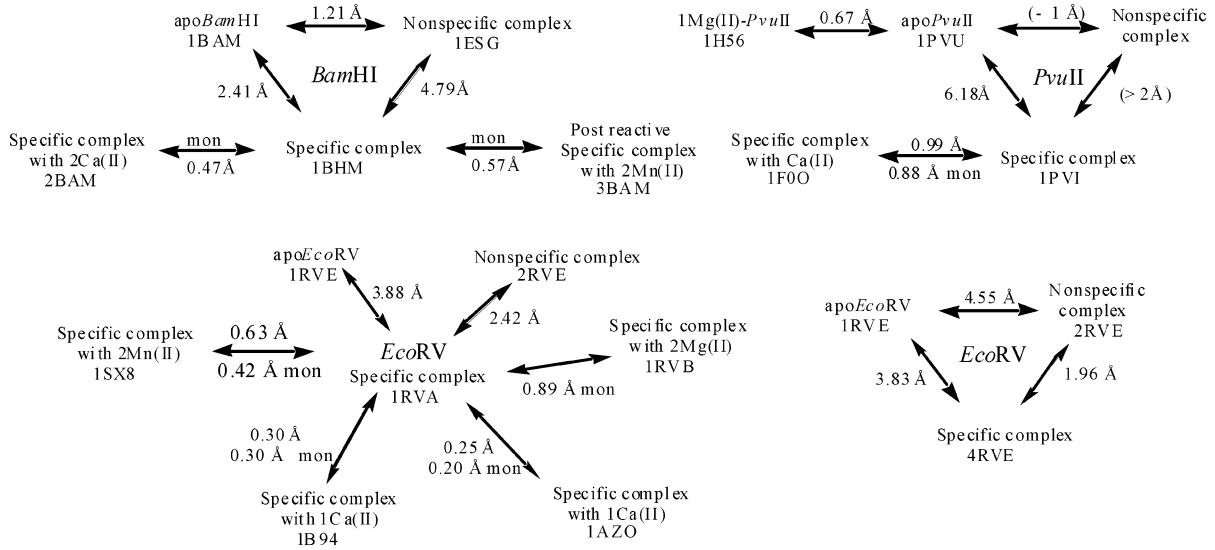

FIGURE 6: Comparison of restriction enzyme complex structures. C_α rms deviations between the specified structures and PDB entries are given. PDB entry 4RVE was edited to contain only two subunits arranged as a homodimer. *Eco*RV−metal−DNA structures were compared to PDB entry 1RVA, which is of higher resolution and shares a space group with these structures. Where appropriate, rmsd values obtained with one monomer (mon) were included to facilitate comparison to PDB entries 2BAM, 3BAM, and 1RVB, all of which feature metal ions in only one subunit. Parenthetical values are those predicted on the basis of overlap analysis of NMR spectra.

differences in nuclear environments between Ca(II)- and Mg(II)-supported complexes could exist, but that DNA still exhibits the most dramatic effects on enzyme conformation. Whatever the source of the spectral differences, this is an ideal issue for further exploration via resonance assignments (see below). This information can be used to quantitatively correlate chemical shift changes with differences in structure.

Conformation and Sequence Recognition. In addition to the general phenomenon of DNA binding, we must also consider the conformational basis of sequence discrimination. Despite the general interest in understanding the basis of sequence recognition, there are only two systems for which structural comparisons between cognate and noncognate complexes can be made. Structures of the free *Bam*HI enzyme (PDB entry 1BAM) (*50*) and the enzyme in complex with both cognate (PDB entry 1BHM) (*45*) and noncognate DNAs (PDB entry 1ESG) (*13*) have been published. In this system, the free enzyme and the nonspecific DNA complex are remarkably similar, sharing an open configuration about the DNA binding interface. The rmsd between the C_α atoms of these structures is only 1.21 Å (Figure 6). Binding of cognate DNA results in perceptible conformational changes and a more closed configuration. The C_α rmsd between noncognate and cognate complexes is a more substantial 4.79 Å.

The other system for which there are informative reference structures is *Eco*RV endonuclease. A C_α rmsd of 4.55 Å separates the structures of the free enzyme (PDB entry 1RVE) from that of the noncognate complex (PDB entry 2RVE) (*1*, *12*). Between noncognate (PDB entry 2RVE) and cognate complexes (PDB entries 1RVA and 4RVE), the C_α rmsd is smaller (<2.5 Å). This illustrates a greater similarity between nonspecific and specific complexes than is observed for *Bam*HI complexes. In the *Pvu*II system, an open apoenzyme structure [PDB entry 1PVU (*21*)] also closes upon binding cognate DNA [PDB entry 1PVI (*20*)]. Indeed, the C_α rmsd between these structures is 6.18 Å. Since there is no published structure of a nonspecific *Pvu*II complex, other structural comparisons cannot be made in this fashion.

The work described here provides an opportunity to establish correlations between crystal and solution structural data for the *Pvu*II system. These can in turn be applied to structures for which there is no crystallographic information. NMR spectral overlap between the *Pvu*II apoenzyme and the enzyme−Mg(II) complex is high (80%), and the C_α rmsd is 0.67 Å (Figure 6). Analogously, NMR spectral overlap between the enzyme−Ca(II) and enzyme−Ca(II)−noncognate complex is fairly high, and the overlap between enzyme−Ca(II)−cognate and enzyme−Ca(II)−noncognate complexes is very small. Therefore, a smaller C_α rmsd is anticipated between the apoenzyme and the nonspecific complex than between the cognate and noncognate complexes. This pattern indicates that *Pvu*II endonuclease behavior is more similar to that of *Bam*HI than that of the *Eco*RV system, where the C_α rmsd between cognate and noncognate complexes is more modest. While it is simple and should be applied conservatively, this kind of analysis provides important information about restriction enzyme conformational behavior in solution. Moreover, it demonstrates the power of NMR spectroscopy in quickly obtaining valuable conformational information for a fairly large, complex system.

The work of von Hippel (*3*) and others recently reviewed (*1*, *51*) have led to a model of one-dimensional diffusion or sliding, in which a restriction enzyme first binds large DNAs nonspecifically and then proceeds along the double helix until it reaches its cognate sequence. In this model, the enzymes bind noncognate DNA with an open conformation, which facilitates movement. Upon reaching the cognate sequence, the enzyme undergoes a conformational change to a more closed conformation that is unique to cognate complexes. Indeed, recent NMR studies of the DNA binding domain of *lac* repressor illustrate this elegantly (*17*). The spectral data presented here provide further support for this model.

Potential of Applying NMR Spectroscopy to Restriction Enzymes. Through this and earlier studies, we have established that unique and important information about restriction enzymes can be obtained via NMR spectroscopy. HSQC

spectra of *Pvu*II endonuclease are of high quality and provide significant insights. These data can also be used to assess the feasibility of more detailed experiments.

A special consideration in planning additional experiments is the size problem. Nuclei in large particles have short relaxation times, which are manifested as increased line widths, which in turn limit the resolution of information in NMR spectra. While restriction enzymes are small compared to many nucleic acid enzymes, they are large systems by NMR standards. The *Pvu*II endonuclease monomer is only 18 kDa, but the enzyme is homodimeric. With the addition of an oligonucleotide, experiments are performed on a particle that is approximately 40 kDa in size, a molecular mass which is on the high end of proteins for which total resonance assignments are relatively routine (*52*, *53*). Interest in the structural behavior of large proteins has led to many recent advances; the application of ultrahigh field magnets, specialized labeling techniques, and TROSY have all extended the size limit (*16*). There are a number of significantly larger proteins for which total sequence assignments have been obtained (*54*, *55*).

The most obvious extension of this work is sequence specific resonance assignments. This would increase the interpretability in HSQC spectra, permitting us to describe what parts of the enzyme structure are perturbed and to what extent. Assignments are typically accomplished with a series of triple-resonance experiments in which through-bond coupling is used to establish sequential connectivities (*56*). HNCO spectra of *Pvu*II endonuclease, which measure coupling between the carbonyl ^{13}C of the *i*th residue and the amide ^{15}N of the residue $i + 1$, are of very high quality with 16 scan acquisitions. However, a preliminary HNCA experiment, which correlates $^{13}C_\alpha(i, i - 1)-^{15}N(i)-NH(i)$ resonances, indicates that C_α relaxation is quite fast for *Pvu*II endonuclease (data not shown). This complication is common for larger proteins, and it compromises the quality of backbone spectra needed for assignments. This issue is typically addressed with deuteration, which eliminates dipolar relaxation of this nucleus by nearby protons (*57*). Another innovation which targets larger proteins is TROSY (transverse relaxation optimized spectroscopy). This modification of existing pulse sequences uncouples mechanisms of relaxation responsible for line broadening in large molecules (dipole−dipole coupling and chemical shift anisotropy) (*30*). In our experience, *Pvu*II spectra respond well to TROSY, thus increasing spectral interpretability. This bodes well for the application of higher fields (≥600 MHz), for which TROSY is designed. Thus, while resonance assignments will be a significant undertaking, the application of these newer techniques should make this feasible.

In addition to characterizing enzyme conformational changes associated with metal ion and DNA binding events, NMR spectroscopy could can be applied to other worthy issues. For example, restriction enzymes exhibit "star activity", a relaxation of sequence specificity which can be induced with pH, salt, organic solvents, or metal ions (*58*, *59*). While some studies have addressed this issue thermodynamically (*60*, *61*), the structural basis of this phenomenon is not well understood. We speculate that there is a conformational basis for this behavior, and it may be possible to explore this via NMR spectroscopy. Second, mutagenesis studies suggest that the coupling between conformation and catalysis is very strong (*62*, *63*). NMR spectroscopy would be useful in elucidating these relationships. Finally, this technique is currently the best means of observing molecular motion on multiple time scales (*64*). It is becoming increasingly clear that conformational dynamics are important aspects of macromolecular function, and nucleic acid binding proteins are proving to be no exception (*17*). As proteins which bind a dynamic polymer like DNA with remarkable specificity and control, restriction enzymes are valuable model systems in which to explore this relationship.

ACKNOWLEDGMENT

Many thanks to my group for assistance with protein purification, to Patricia LiWang and Nancy Horton for helpful discussions, and to the reviewers for their constructive comments.

REFERENCES

1. Pingoud, A., and Jeltsch, A. (2001) Structure and function of type II restriction endonucleases, *Nucleic Acids Res. 29*, 3705−3727.
2. Pabo, C., and Sauer, R. T. (1992) Transcription factors: Structural families and principles of DNA recognition, *Annu. Rev. Biochem. 61*, 1053−1095.
3. von Hippel, P. H., and Berg, O. G. (1989) Facilitated target location in biological systems, *J. Biol. Chem. 264*, 675−678.
4. von Hippel, P. H. (1994) Protein-DNA recognition: New perspectives and underlying themes, *Science 263*, 769−770.
5. Spolar, R. S., and Record, M. T. (1994) Coupling of local folding to site-specific binding of proteins to DNA, *Science 263*, 777−784.
6. Nadassy, K., Wodak, S., and Janin, J. (1999) Structural features of protein-nucleic acid recognition sites, *Biochemistry 38*, 1999−2017.
7. Halford, S. E., and Johnson, N. P. (1983) Single turnovers of the *Eco*RI restriction endonuclease, *Biochem. J. 211*, 405−415.
8. Jen-Jacobson, L., Engler, L., Ames, J., Kurpiewski, M., and Grigorescu, A. (2000) Thermodynamic parameters of specific and nonspecific protein-DNA binding, *Supramol. Chem. 12*, 143−160.
9. Baldwin, G. S., Vipond, I. B., and Halford, S. E. (1995) Rapid reaction analysis of the catalytic site of the *Eco*RV restriction endonuclease, *Biochemistry 34*, 705−714.
10. Jen-Jacobson, L. (1995) Structural-perturbation approaches to thermodynamics of site-specific protein-DNA interactions, *Methods Enzymol. 259*, 305−344.
11. Pingoud, A. (2004) *Restriction Endonucleases*, Vol. 14, Springer, Berlin.
12. Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., and Wilson, K. S. (1993) The crystal structure of *Eco*RV endonuclease and of its complexes with cognate and noncognate DNA fragments, *EMBO J. 12*, 1781−1795.
13. Viadiu, H., and Aggarwal, A. (2000) Structure of *Bam*HI bound to nonspecific DNA: A model for DNA sliding, *Mol. Cell 5*, 889−895.
14. Kalodimos, C., Boelens, R., and Kaptein, R. (2004) Toward an integrated model of protein-DNA recognition as inferred from NMR studies on the *lac* repressor system, *Chem. Rev. 104*, 3567−3586.
15. Cavanagh, J., Fairbrother, W., Palmer, A., and Skelton, N. (1996) *Protein NMR Spectroscopy: Principles and Practice*, Academic Press, New York.
16. Wuthrich, K. (1998) The second decade: Into the third millenium, *Nat. Struct. Biol.* (NMR Suppl.), 492−495.
17. Kalodimos, C., Biris, N., Bonvin, A., Levandoski, M., Geuenneugues, M., Boelens, R., and Kaptein, R. (2004) Structure and flexibility adaptation in nonspecific and specific protein-DNA complexes, *Science 305*, 386−389.
18. Gingeras, T. R., Greenough, L., Schildkraut, I., and Roberts, R. J. (1981) Two new restriction endonucleases from Proteus vulgaris, *Nucleic Acids Res. 9*, 4525−4536.
19. Conlan, L. H., and Dupureur, C. M. (2002) Dissecting the metal ion dependence of DNA binding by *Pvu*II endonuclease, *Biochemistry 41*, 1335−1342.

20. Cheng, X., Balendiran, K., Schildkraut, I., and Anderson, J. E. (1994) Structure of *Pvu*II endonuclease with cognate DNA, *EMBO J. 13*, 3927−3935.
21. Athanasiadis, A., Vlassi, M., Kotsifaki, D., Tucker, P. A., Wilson, K. S., and Kokkinidis, M. (1994) Crystal structure of *Pvu*II endonuclease reveals extensive structural homologies to *Eco*RV, *Struct. Biol. 1*, 469−475.
22. Dupureur, C. M., and Hallman, L. M. (1999) Effects of divalent metal ions on the activity and conformation of native and 3-fluorotyrosine-*Pvu*II endonucleases, *Eur. J. Biochem. 261*, 261−268.
23. Dupureur, C. M., and Dominguez, M. A., Jr. (2001) The PD.... (D/E)XK motif in restriction enzymes: A link between structure and conformation, *Biochemistry 40*, 387−394.
24. Holmquist, B. (1988) Elimination of Adventitious Metals, *Methods Enzymol. 158*, 6−12.
25. Balendiran, K., Bonventre, J., Knott, R., Jack, W., Benner, J., Schildkraut, I., and Anderson, J. E. (1994) Expression, purification, and crystallization of restriction endonuclease *Pvu*II with DNA containing its recognition site, *Proteins: Struct., Funct., Genet. 19*, 77−79.
26. Wagner, F. W. (1988) Preparation of metal-free enzymes, *Methods Enzymol. 158*, 21−32.
27. Jen-Jacobson, L., Kurpiewski, M., Lesser, D., Grable, D., Boyer, H. W., Rosenberg, J. M., and Greene, P. J. (1983) Coordinate ion pair formation between *Eco*RI endonuclease and DNA, *J. Biol. Chem. 258*, 14638−14646.
28. José, T. J., Conlan, L. H., and Dupureur, C. M. (1999) Quantitative evaluation of metal ion binding to *Pvu*II restriction endonuclease, *J. Biol. Inorg. Chem. 4*, 814−823.
29. Roberts, G. C. K. (1993) *NMR of Macromolecules*, IRL, New York.
30. Pervushin, K., Riek, R., Wider, G., and Wuthrich, K. (1997) Attenuated T_2 relaxation by mutual cancellation of dipole−dipole coupling and chemical shift anistropy indicates an avenue to NMR structures of very large biological macromolecules in solution, *Proc. Natl. Acad. Sci. U.S.A. 94*, 12366−12371.
31. Jeltsch, A., Alves, J., Maass, G., and Pingoud, A. (1992) On the catalytic mechanism of EcoRI and EcoRV, *FEBS Lett. 304*, 4−8.
32. Dupureur, C. M., and Conlan, L. H. (2000) A catalytically deficient active site variant of *Pvu*II endonuclease binds Mg(II) ions, *Biochemistry 39*, 10921−10927.
33. Spyridaki, A., Matzen, C., Lanio, T., Jeltsch, A., Simoncsits, A., Athanasiadis, A., Scheuring-Vanamee, E., Kokkinidis, M., and Pingoud, A. (2003) Structural and biochemical characterization of a new Mg^{2+} binding site near Tyr94 in the restriction enzyme *Pvu*II, *J. Mol. Biol. 331*, 395−406.
34. Conlan, L. H., and Dupureur, C. M. (2002) Multiple metal ions drive DNA association by *Pvu*II endonuclease, *Biochemistry 41*, 14848−14855.
35. King, J., Bowen, L., and Dupureur, C. M. (2004) Binding and conformational analysis of phosphoramidate-restriction enzyme interactions, *Biochemistry 43*, 8551−8559.
36. Zhong, X., Patel, S. S., Werneburg, B. G., and Tsai, M. D. (1997) DNA polymerase β: Multiple conformational changes in the mechanism of catalysis, *Biochemistry 36*, 11891−11900.
37. Ahn, J., Kraynov, V. S., Zhong, X., Werneburg, B. G., and Tsai, M. D. (1998) DNA polymerase β: Effects of gapped DNA substrates on dNTP specificity, fidelity, processivity and conformational changes, *Biochem. J. 331*, 79−87.
38. Jen-Jacobson, L., Engler, L. E., Lesser, D. R., Kurpiewski, M. R., Yee, C., and McVerry, B. (1996) Structural adaptations in the interaction of *Eco*RI endonuclease with methylated GAATTC sites, *EMBO J. 15*, 2870−2882.
39. Becker, M., Lesser, D., Kurpiewski, M., Baranger, A., and Jen-Jacobson, L. (1988) "Ultraviolet Footprinting" accurately maps sequence-specific contacts and DNA kinking in the *Eco*RI endonuclease−DNA complex, *Proc. Natl. Acad. Sci. U.S.A. 85*, 6247−6251.
40. Lesser, D. R., Kurpiewski, M. R., and Jacobson, L. J. (1990) The energetic basis of specificity in the *Eco*RI endonuclease−DNA interaction, *Science 250*, 776−784.
41. Rosenberg, J. M. (1991) Structure and function of restriction endonucleases, *Curr. Opin. Struct. Biol. 1*, 104−113.
42. Kostrewa, D., and Winkler, F. K. (1995) Mg^{2+} binding to the active site of *Eco*RV endonuclease: A crystallographic study of complexes with substrate and product DNA at 2 Å resolution, *Biochemistry 34*, 683−696.
43. Lagunavicius, A., Grazulis, S., Balciunaite, E., Vainius, D., and Siksnys, V. (1997) DNA binding specificity of *Mun*I restriction endonuclease is controlled by pH and calcium ions: Involvement of active site carboxylate residues, *Biochemistry 36*, 11093−11099.
44. Vipond, I. B., and Halford, S. E. (1995) Specific DNA recognition by *Eco*RV restriction endonuclease induced by calcium ions, *Biochemistry 34*, 1113−1119.
45. Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., and Aggarwal, A. K. (1995) Structure of *Bam*HI endonuclease bound to DNA: Partial folding and unfolding on DNA binding, *Science 269*, 656−663.
46. Horton, J. R., and Cheng, X. (2000) *Pvu*II endonuclease contains two calcium ions in active sites, *J. Mol. Biol. 300*, 1049−1056.
47. Horton, N. C., and Perona, J. J. (2004) DNA Cleavage by *Eco*RV endonuclease: Two metal ions in three metal ion binding sites, *Biochemistry 43*, 6841−6857.
48. Perona, J. J., and Martin, A. M. (1997) Conformational Transitions and Structural Deformability of *Eco*RV Endonuclease Revealed by Crystallographic Analysis, *J. Mol. Biol. 273*, 207−225.
49. Thomas, M., Brady, R., Halford, S. E., Sessions, R., and Baldwin, G. S. (1999) Structural analysis of a mutational hot spot in the *Eco*RV restriction endonuclease: A catalytic role for a main chain carbonyl group, *Nucleic Acids Res. 27*, 3438−3445.
50. Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., and Aggarwal, A. K. (1994) Structure of restriction endonuclease *Bam*HI at 1.95 Å resolution by MAD analysis, *Structure 2*, 439−452.
51. Jeltsch, A., and Urbanke, C. (2004) Sliding or hopping? How restriction enzymes find their way on DNA, in *Nucleic Acids and Molecular Biology* (Pingoud, A., Ed.) pp 95−110, Springer-Verlag, Berlin.
52. Clore, G., and Gronenborn, A. (1998) NMR structure determination of proteins and protein complexes larger than 20 kDa, *Curr. Opin. Struct. Biol. 2*, 564−570.
53. Arrowsmith, C. H., and Wu, Y.-S. (1998) NMR of large (>25 kDa) proteins and protein complexes, *Prog. NMR Spectrosc. 32*, 277−286.
54. Kelly, M., Ball, L., Krieger, C., Yu, Y., Fischer, M., Schiffman, S., Schmieder, P., Kuhne, R., Bermel, W., Bacher, W., Richter, G., and Oschkinat, H. (2001) The NMR structure of the 47-kD dimeric enzyme 3,4-diydroxy-2-butanone-4-phosphate synthase and ligand binding studies reveal the location of the active site, *Proc. Natl. Acad. Sci. U.S.A. 98*, 13025−13030.
55. Turgarinov, V., Muhandiram, R., Ayed, A., and Kay, L. (2002) Four-dimensional NMR spectroscopy of a 723-residue protein: Chemical shift assignments and secondary structure of malate synthase G, *J. Am. Chem. Soc. 124*, 10025−10035.
56. Clore, G. M., and Gronenborn, A. M. (1994) Multidimensional heteronuclear nuclear magnetic resonance of proteins, *Methods Enzymol. 239*, 349−363.
57. Gardner, K. H., and Kay, L. E. (1998) The use of ^{2}H, ^{13}C, ^{15}N multidimensional NMR to study the structure and dynamics of proteins, *Annu. Rev. Biophys. Biomol. Struct. 27*, 357−406.
58. Polisky, B., Greene, P., Garfin, D. E., McCarthy, B. J., Goodman, H. M., and Boyer, H. W. (1975) Specificity of substrate recognition by the *Eco*RI restriction endonuclease, *Proc. Natl. Acad. Sci. U.S.A. 72*, 3310−3314.
59. Taylor, J. D., and Halford, S. E. (1989) Discrimination between DNA sequences by the *Eco*RV restriction endonuclease, *Biochemistry 28*, 6198−6207.
60. Sidorova, N. Y., and Rau, D. C. (1996) Differences in water release for the binding of *Eco*RI to specific and nonspecific sequences, *Proc. Natl. Acad. Sci. U.S.A. 93*, 12272−12277.
61. Robinson, C. R., and Sligar, S. G. (1998) Changes in solvation during DNA binding and cleavage are critical to altered specificity of the *Eco*RI endonuclease, *Proc. Natl. Acad. Sci. U.S.A. 95*, 2186−2191.
62. Nastri, H. G., Evans, P. D., Walker, I. H., and Riggs, P. D. (1997) Catalytic and DNA binding properties of *Pvu*II restriction endonuclease mutants, *J. Biol. Chem. 272*, 25761−25767.
63. Jeltsch, A., Alves, J., Oelgeschlager, T., Wolfes, H., Maass, G., and Pingoud, A. (1993) Mutational analysis of the function of Gln115 in the *Eco*RI restriction endonuclease, a critical amino acid for recognition of the inner thymidine residue in the sequence -GAATTC- and for coupling specific DNA binding to catalysis, *J. Mol. Biol. 229*, 221−234.
64. Kay, L. (1998) Protein dynamics from NMR, *Nat. Struct. Biol. 5* (Suppl.), 513−517.

BI0473758