Divalent Metal Dependence of Site-Specific DNA Binding by *Eco*RV Endonuclease†

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Received March 8, 1999; Revised Manuscript Received April 29, 1999

ABSTRACT: Measurements of binding equilibria of *Eco*RV endonuclease to DNA, for a series of base-analogue substrates, demonstrate that expression of sequence selectivity is strongly enhanced by the presence of Ca^{2+} ions. Binding constants were determined for short duplex oligodeoxynucleotides containing the cognate DNA site, three cleavable noncognate sites, and a fully nonspecific site. At pH 7.5 and 100 mM NaCl, the full range of specificity from the specific (tightest binding) to nonspecific (weakest binding) sites is 0.9 kcal/mol in the absence of metal ions and 5.8 kcal/mol in the presence of Ca^{2+}. Precise determination of binding affinities in the presence of the active Mg^{2+} cofactor was found to be possible for substrates retaining up to 1.6% of wild-type activity, as determined by the rate of phosphoryl transfer. These measurements show that Ca^{2+} is a near-perfect analogue for Mg^{2+} in binding reactions of the wild-type enzyme with DNA base-analogue substrates, as it provides identical $\Delta\Delta G°_{bind}$ values among the cleavable noncognate sites. Equilibrium dissociation constants of wild-type and base-analogue sites were also measured for the weakly active *Eco*RV mutant K38A, in the presence of either Mg^{2+} or Ca^{2+}. In this case, Ca^{2+} allows expression of a greater degree of specificity than does Mg^{2+}. $\Delta\Delta G°_{bind}$ values of K38A toward specific versus nonspecific sites are 6.1 kcal/mol with Ca^{2+} and 3.9 kcal/mol with Mg^{2+}, perhaps reflecting metal-specific conformational changes in the ground-state ternary complexes. The enhancement of binding specificity provided by divalent metal ions is likely to be general to many restriction endonucleases and other metal-dependent nucleic acid-modifying enzymes. These results strongly suggest that measurements of DNA binding affinities for *Eco*RV, and likely for many other restriction endonucleases, should be performed in the presence of divalent metal ions.

Protein engineering studies on restriction endonucleases (and other enzymes) require the rigorous correlation of thermodynamic and kinetic parameters with structural information. If a database of such structure−function correlations is to be useful in providing insights for a successful redesign of catalytic properties, it is essential that the measurements be both consistently interpretable and reflective of the physical and chemical steps as they occur in a true catalytic cycle. One important challenge in applying these principles to restriction endonucleases is that of establishing the experimental design for quantitating DNA binding affinity. Certain divalent metal ions are absolutely required for DNA cleavage by these enzymes (*1*), and it has been argued that their omission provides a simple and effective strategy for blocking the reaction and thus isolating the binding step (*2*). Alternatively, the use of modified ternary complexes which cannot proceed to the transition state has been envisioned. Modifications in the enzyme, in the DNA, and in the type of metal ion are each possible for restriction enzymes, providing a range of opportunities for examining the binding equilibria.

Early experiments with *Eco*RI, *Bam*HI, and *Rsr*I endonucleases demonstrated, as expected, specific DNA binding in the absence of metal ions (*3*−*6*). However, studies with other restriction enzymes, beginning with *Eco*RV (*7*), showed an apparent lack of binding specificity under these conditions. For example, in the absence of metal ions at pH 7.5 *Mun*I endonuclease does not show a specific gel shift with 160 bp DNA restriction fragments, regardless of whether these substrates contain the specific site. However, lowering the pH to 6.5, replacement of active site acidic residues with alanine, and addition of the inactive cofactor Ca^{2+} each result in the observation of shifted specific DNA fragments at relatively lower protein concentrations. Although only semiquantitative in nature (no K_d values were reported), these studies support the notion that the alterations improve the ability of the enzyme to discriminate among DNA sequences (*8*, *9*). Specificity thus appears to be enhanced by shielding closely juxtaposed negative charges on the protein and DNA. This can be accomplished by lowering pH, including divalent metal ions, or removing the protein acidic groups. Consistent with this view, crystal structures of *Eco*RV, *Bam*HI, and *Bgl*I endonucleases reveal metal ions bound in the active sites between enzyme carboxylates and the scissile DNA phosphates (*10*−*12*). In similar experiments, *Cfr*10I and *Pvu*II also manifest apparently improved binding specificity in the presence of divalent metals (*13*, *14*). *Taq*I and *Cfr*9I are

† This work was supported by NIH Grant GM53763 and ACS-PRF Grant 33381-AC4 (to J.J.P.).

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10.1021/bi9905359 CCC: $18.00
Published on Web 06/04/1999

further examples of enzymes which appear not to bind specifically in the absence of these cofactors (*15*, *16*).

These studies suggest that measurements of binding equilibria for restriction enzymes might best be made in the presence of Ca^{2+}, as an analogue which mimics the electrostatic properties of active metal ions such as Mg^{2+} and Mn^{2+}, but which does not support catalysis (*17*). However, a number of important questions have remained unresolved. First, the very observation that Ca^{2+} ions do not foster DNA cleavage raises the possibility that differences in certain properties, such as ionic radius or preferred coordination geometry, might also render them poor substitutes in the binding reaction. Second, the question has been raised of whether the distinction in metal ion requirements for binding specificity, between *Eco*RI-like and *Eco*RV-like restriction enzymes, is truly indicative of a suggested fundamental division into separate mechanistic classes (*18*). Evidence that the differences in metal ion requirements are instead primarily due to differences in solution conditions and choices of substrates has been presented (*19*). Third, a quantitative assessment of the degree to which specificity is truly enhanced by Ca^{2+} has been lacking, as has exploration of whether such enhancement might be general across the enzyme family, including those enzymes for which binding specificity in the absence of metal ions *is* readily detected (*3*−*6*).

In this study, we investigate several of these questions through the use of *Eco*RV endonuclease as the model system. A number of studies exploring the metal ion dependence of DNA binding specificity in *Eco*RV have been previously carried out. Several different experiments in which diverse methodologies were employed, in which divalent cations were omitted, each suggested that the enzyme binds all DNA sequences with equal affinity (*7*, *20*, *21*). However, this finding was challenged by the demonstration that specific binding in the absence of Mg^{2+} is strongly pH-dependent (*19*), and by the detection of specific binding by resonance Raman methods in the absence of metals (*22*). It has also been shown that Ca^{2+} increases the affinity for binding to the specific site and to two noncleavable DNA thio-analogue sites by 200−700-fold, with the effect originating from a decreased rate of dissociation of the protein−DNA complex (*19*). By contrast, little enhancement of binding affinity was found for the thio analogues in the presence of Mg^{2+}. These data were interpreted to mean that Ca^{2+} produces an aberrant ternary complex, and is thus a poor model for the normal Mg^{2+} cofactor (*19*). However, the observation that Mg^{2+} and Ca^{2+} occupy very similar positions in the crystal structures of the ternary complexes suggests instead that the analogy between these metals should be quite good (*11*, *23*).

In light of the ambiguities, and in view of the importance to continued protein engineering studies of resolving these questions, we have carried out a new investigation of the divalent metal dependence of specific binding by *Eco*RV. The goals of this study were 4-fold: (i) to provide a quantitative measure of the extent to which DNA binding selectivities for a well-defined set of short oligodeoxynucleotide substrates differ when measured by omitting the metal entirely versus including Ca^{2+}; (ii) to directly assess the suitability of Ca^{2+} as an analogue for Mg^{2+}, by studying *active* modified complexes with cleavage rates sufficiently slow to allow binding measurements in the presence of Mg^{2+}; (iii) to evaluate whether Mg^{2+}, as a metal ion which can promote catalysis, is able to improve the DNA binding selectivity of the enzyme; and (iv) to examine the metal-dependent binding behavior of an enzyme mutant deficient in the coupling of DNA binding to catalysis. These thermodynamic measurements provide significant new information relative to the previous studies (*7*, *19*−*22*, *24*, *25*). We show definitively that the sequence specificity of binding for short oligodeoxynucleotide substrates is dramatically enhanced by Ca^{2+} relative to the condition where metal is absent. Under our experimental conditions (pH 7.5 and 100 mM NaCl), the full range of binding specificity manifested by *Eco*RV is 10^4-fold in the presence of Ca^{2+} and only 5-fold in its absence. Further, we demonstrate by direct comparisons of Mg^{2+} and Ca^{2+} binding reactions that Ca^{2+} has the capacity to perform as a near-perfect analogue for the active Mg^{2+}. These comparisons also reveal that Mg^{2+} can provide at least 100-fold enhancement of the DNA binding specificity. However, the analogy between Ca^{2+} and Mg^{2+} breaks down to different extents for some modified complexes, likely reflecting metal-specific structural differences at the protein−DNA interface. These findings have significant implications in the study of other metal-dependent nucleic acid-modifying enzymes, and may be helpful in considering the design of protein engineering studies for other systems as well.

MATERIALS AND METHODS

Preparation of the K38A Mutant. The K38A mutant of *Eco*RV was constructed by site-directed mutagenesis using PCR methodologies. Expression plasmid *p*BSRV encoding the wild-type *Eco*RV endonuclease gene was used as a template (*26*). A wild-type primer at the unique *Bam*HI site and a second primer at the unique *Pst*I site, carrying the lysine to alanine substitution (AAA to CGA), were used in a standard polymerase chain reaction (PCR) (at 50 μM dNTPs). The 510 bp fragment synthesized in this reaction was digested with *Pst*I and *Bam*HI, phenol extracted, ethanol precipitated, and ligated into a *p*BSRV vector fragment generated via double digestion with *Pst*I and *Bam*HI followed by gel purification. The ligation reaction mixture was transformed into competent *Escherichia coli* MM294 (*endI*$^-$, *pro*$^-$, *thi*$^-$, $r_k^-m_k^+$) cells containing the *p*MetB plasmid, which encodes the *Eco*RV methyltransferase and kanamycin resistance genes (*27*). Ligation mixtures were plated directly onto prewarmed LB plates containing 50 μg/mL ampicillin, without kanamycin. Colonies were then inoculated at 37 °C in LB medium containing 50 μg/mL ampicillin and 20 μg/mL kanamycin. More than 90% of the colonies which were inoculated grew normally, indicating that the *p*MetB plasmid had been retained despite the absence of kanamycin on the plates. Plasmid DNA was isolated using the PlasmidPURE kit (Sigma), and the sequence of the entire *Eco*RV K38A gene was confirmed.

Preparation of Enzymes and DNA Substrates. Wild-type and K38A mutant *Eco*RV enzymes were expressed in *E. coli*, purified to homogeneity by a two-column procedure, and stored as described previously (*23*, *28*).

The set of DNA oligonucleotides that were used comprised variations of the following non-self-complementary 16-mer: GGGAAAGA**TA**TCTTGG (**TA**). The oligonucleotides contained modifications to the center two base pairs of the

*Eco*RV recognition sequence and are designated by the nucleotides replacing the TA center step in both strands: **UA**, **CI**, and **CG** (I, inosine; U, deoxyuracil). A fifth nonspecific substrate was designed by incorporating the *Eco*RV recognition sequence backward within the same flanking sequence: GGGAAACTATAGTTGG (**Nsp**). DNA oligonucleotides were purchased from the Midland Certified Reagent Co. and from Integrated DNA Technologies, Inc. The DNA was purified by HPLC and radiolabeled as described previously (*28*). T4 polynucleotide kinase was purchased from New England Biolabs. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham.

Equilibrium Gel Shift Binding Assays. All thermodynamic association constants (K_a) measured in the absence of divalent metal ions, for both wild-type and K38A *Eco*RV enzymes, were determined by direct gel shift. DNA concentrations at least 90-fold lower than the dissociation constant were used, and enzyme concentrations were titrated over a 100-fold range. The binding buffer for the gel shifts contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 500 μg/mL BSA, 10 mM EDTA, and 10 μM β-mercaptoethanol. The reaction mixtures were incubated at 22 °C for 15 min. Gel loading buffer without dye was added to each reaction mixture (such that final concentrations of all binding buffer components remained the same with glycerol added to a final concentration of 11%), immediately before loading onto a running 10% polyacrylamide gel. The gel running buffer is composed of 5.4 mM diethylbarbituric acid and 46 mM Tris, and was adjusted to pH 7.0 with HCl (*29*). The gels were run at 220 V and 4 °C for 75 min. Autoradiography and densitometry of the gels were performed with the Storm 840 phosphorimager (Molecular Dynamics). Both the shifted and unshifted bands were quantitated to determine the relative amount of complex formed at each enzyme concentration. The association constants were determined by fitting these data to a standard hyperbolic curve using Kaleidagraph. All measurements were taken in triplicate. The reported K_a is the average of the three values, and the error is the standard deviation from the mean.

To perform gel shifts in the presence of metal ions, the binding buffer contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 500 μg/mL BSA, 10 mM β-mercaptoethanol, and either 10 mM $CaCl_2$ or 10 mM $MgCl_2$. The gel running buffer contained 89 mM Tris-borate, 89 mM boric acid, and either 2 mM $CaCl_2$ or 2 mM $MgCl_2$. The pH of this buffer is 8.5. A running buffer containing Ca^{2+} ions at pH 7.0 was also prepared for one of the control experiments. It contains 5.4 mM diethylbarbituric acid, 46 mM Tris-HCl (pH 7.0), and 10 mM Ca^{2+}. The gels were run at 220 V and 4 °C for 105 min. All other conditions for the binding reactions, and for running and analyzing the gels, were identical to those for the gel shifts performed in the absence of divalent metal.

Most of the substrate binding affinities in the presence of Ca^{2+} or Mg^{2+} were measured by competition gel shift. This method was necessary for those substrates with low binding affinities, because in direct gel shifts such weakly bound complexes often dissociate in the gel. Competition gel shifts were also much less technically demanding than direct gel shifts for the very high-affinity substrates, because much higher concentrations of DNA could be used with substantial reduction of exposure times (*28*). To perform competition binding assays, enzyme and radiolabeled **TA**, **UA**, or **CI** substrate DNA were equilibrated at a fixed concentration of each component, under conditions at which 80% of the labeled DNA was shifted. Unlabeled competitor DNA (**UA**, **CG**, or **Nsp**) was then titrated in at concentrations from 0 to 2 μM. All other conditions were as described above. For all reactions, the K_a of either the competitor DNA or the labeled DNA was known. Therefore, the unknown K_a could be determined by fitting the data to an equation describing the equilibrium between the enzyme and the two substrates (*30*, *31*). All measurements were taken in triplicate. The reported K_a is the average of the three values, and the error is the standard deviation from the mean.

Measurements of binding affinities for the **TA**, **UA**, **CI**, and **CG** substrates in the presence of Ca^{2+} for wild-type *Eco*RV have been previously reported (*28*). In that study, the binding affinity toward **CI** was measured by direct gel shift. This known value was used here to determine the K_a of wild-type *Eco*RV toward **Nsp** in the presence of Ca^{2+}, using unlabeled **Nsp** as the competitor (**CI**/**Nsp**). The determinations of Mg^{2+}-dependent binding affinities to wild-type *Eco*RV were carried out as follows. The **CI** substrate was assessed by direct gel shift, and **CI** was then used in competition gel shifts with **UA**, **CG**, and **Nsp**. **CG** and **Nsp** were both used as the unlabeled competitor with labeled **CI**, whereas in the Mg^{2+}-dependent **UA** reaction, the **CI** substrate was used as the unlabeled competitor. Measurement of the level of Mg^{2+}-dependent binding of wild-type enzyme to **TA** is not possible because the substrate is rapidly cleaved.

A similar strategy was used for measuring metal-dependent binding affinities for binding to the K38A mutant. The **CI** substrate was again assessed by direct gel shift for both Mg^{2+} and Ca^{2+} reactions. This substrate was then used as the labeled DNA in competition gel shifts to determine the K_a values of **CG** and **Nsp** (**CI**/**CG** and **CI**/**Nsp**) in the presence of each metal. **CG** was then used as unlabeled competitor in competition gel shifts with labeled **TA** and **UA** to determine the K_a values of these latter two substrates (**TA**/**CG** and **UA**/**CG**), for Ca^{2+} shifts. The K_a of **TA** for Mg^{2+} shifts was determined by using it as the labeled DNA with **CG** as the unlabeled competitor (**TA**/**CG**). The K_a of **UA** for Mg^{2+} shifts was determined by using **UA** as the unlabeled competitor in gel shifts with labeled **TA** (**TA**/**UA**). The K_a of K38A for **TA** with Mg^{2+} was also determined with **CI** as the unlabeled competitor (**TA**/**CI**) as a control, to verify consistency regardless of which pairs were used for the competition gel shifts. The equilibrium association constants determined with different pairs were very similar [$K_a = (4.2 \pm 1.0) \times 10^{10}$ M^{-1}; see also Table 1]. We have also previously shown that direct and competition gel shifts performed on the same DNA substrate give identical K_a values to within 20% error (*28*).

RESULTS

Dependence of the Binding Specificity on Divalent Metal Ions. Binding affinities of *Eco*RV were measured by gel retardation analysis, for a related set of five DNA sites incorporated into matched 16-nucleotide duplex oligodeoxynucleotide substrates (Figure 1). For each substrate, measurements were made both in the absence of metal ions and in the presence of Ca^{2+} as an analogue for the active metals Mg^{2+}, Mn^{2+}, and Co^{2+} (*32*). The substrates include the

Table 1: Equilibrium Association Constants for Binding of Wild-Type *Eco*RV to Cognate and Base-Analogue Sites

substrate	K_a without metal (M^{-1})	$\Delta\Delta G$ without metal (kcal/mol)[a]	$K_a(Ca^{2+})$ (M^{-1})[c]	$\Delta\Delta G(Ca^{2+})$ (kcal/mol)[a]	$K_a(Mg^{2+})$ (M^{-1})	$\Delta\Delta G(Mg^{2+})$ (kcal/mol)[b]
GA**TA**TC	$(8.3 \pm 0.2) \times 10^8$	0.00	$(8.3 \pm 4.2) \times 10^{12}$	0.0	–	–
GA**UA**TC	$(1.0 \pm 0.1) \times 10^9$	−0.11	$(3.0 \pm 0.8) \times 10^{11}$	1.9	$(8.3 \pm 1.0) \times 10^{10}$	0.76
GA**CI**TC	$(8.3 \pm 2.2) \times 10^8$	0.00	$(1.8 \pm 0.1) \times 10^{10}$	3.6	$(7.6 \pm 1.1) \times 10^9$	0.51
GA**CG**TC	$(4.5 \pm 0.6) \times 10^8$	0.36	$(1.1 \pm 0.02) \times 10^9$	5.2	$(3.4 \pm 1.9) \times 10^8$	0.67
CTATAG (**Nsp**)	$(1.8 \pm 0.3) \times 10^8$	0.89	$(4.5 \pm 1.1) \times 10^8$	5.8	$(1.7 \pm 0.6) \times 10^8$	0.58

[a] Free energy differences were calculated from $(K_a)_{WT}/(K_a)_{analogue} = e^{(\Delta\Delta G/RT)}$, with a T of 295 K (*46*). [b] Free energy differences were calculated from $(K_a)_{Ca}/(K_a)_{Mg} = e^{(\Delta\Delta G/RT)}$, with a T of 295 K. [c] From ref *28*.

*Eco*RV cognate sequence GATATC (designated **TA**) and modifications of this sequence with symmetric changes to the center two bases in both DNA strands: GAUATC (**UA**), GACITC (**CI**), and GACGTC (**CG**). These four substrates are each specifically cleaved by *Eco*RV in a blunt-ended fashion at the center base pair step, with Mg^{2+}-dependent rates of phosphoryl transfer at pH 7.5 varying from 70 min^{-1} (**TA**) to 3.1×10^{-5} min^{-1} (**CG**) (*28*). Binding affinities in the presence of Ca^{2+} have also been shown to vary over a wide range ($\Delta\Delta G°_{bind}$ for **TA** vs **CG** = 5.2 kcal/mol; Table 1 and Figure 2; *28*). An additional nonspecific substrate (**Nsp**) was added for this study, to examine the full range of binding affinities available to the enzyme. This DNA site incorporates the recognition sequence in a reverse orientation (CTATAG) so that all of the hydrogen bonding donor and acceptor groups have been interchanged. This may produce the weakest affinity DNA site (*2*). We find that **Nsp** binds with a K_a of 4.5×10^8 M^{-1} in the presence of Ca^{2+}, expanding the dynamic range relative to the specific site to 5.8 kcal/mol (Figures 1 and 2). This represents the best assessment of the full degree of specificity manifested by *Eco*RV at the binding step in the presence of Ca^{2+}, at pH 7.5 and 100 mM NaCl.

Equilibrium binding constants were then determined by gel shift in the absence of divalent metal ions at pH 7.5 (Figures 1 and 2). Under these conditions, the level of binding discrimination between the **TA** and **Nsp** substrates is reduced to only 5-fold (Figure 2). *Eco*RV does retain the ability to discriminate among some of the analogue sites in the absence of metal ions, but the degree of selectivity is severely reduced. The ability to discriminate between **TA**, **UA**, and **CI** is lost altogether, while specificity versus the noncognate, biologically relevant **CG** substrate is decreased to only 2-fold. The binding affinity for the cognate site in the absence of divalent metals is 10^4-fold weaker than in the presence of Ca^{2+}. However, binding to the **CG** and **Nsp** substrates is only 2.5-fold weaker. It is thus clear that the enhancement of specificity arising from the inclusion of Ca^{2+} ions depends almost entirely on tighter binding to the cognate site.

It has been shown that specific binding by *Eco*RV is highly pH-dependent, such that the binding affinity in the absence of divalent metals increases with decreasing pH (*19*). Since the mixtures for the binding reactions in the presence of Ca^{2+} are incubated at pH 7.5, but the gels are run at pH 8.5, it is important to demonstrate that the higher-pH conditions of the gels do not result in complex destabilization. To address this, identical binding reactions at pH 7.5 of *Eco*RV with **CI** substrate and Ca^{2+} were run on two different gels, each incorporating Ca^{2+} in the running buffer. One gel was run at pH 7.0 and the other at pH 8.5. The equilibrium dissociation constants were found to be identical within 30% (data not shown). Therefore, running the Ca^{2+}-containing gels at pH 8.5 does not significantly affect the K_a. In separate control experiments, mixtures for the binding reactions in the absence of metal ions with the 22-mer oligodeoxynucleotide 5′-CTCTTGCGGGATATCGTCCATT were incubated at pH 7.0 and run on parallel gels, also lacking metal ions, at pH 7.0 and pH 6.2. Identical values of 2×10^9 M^{-1} were obtained, as also previously determined with this particular substrate (*19*). We also examined whether the presence of Ca^{2+} within the gel affects the K_a of a reaction performed in the absence of metal ions. The K_a for *Eco*RV binding to **CI** is 40-fold higher in the presence of Ca^{2+} than in the absence of metal ions (Table 1). If the gel conditions used were in fact to influence the equilibrium, then a reaction performed without metal ions and run on a Ca^{2+}-containing gel would be expected to exhibit a significantly higher K_a. To evaluate this possibility, mixtures for identical binding reactions performed with *Eco*RV and **CI** substrate at pH 7.5, in the presence of 10 mM EDTA, were loaded onto three different gels: one lacking metal ions at pH 7.0, a second containing Ca^{2+} at pH 7.0, and a third containing Ca^{2+} at pH 8.5. The three association constants were identical within 30% error (data not shown). We conclude that all the conditions we employed for the diagnostic gels are highly stabilizing, such that the reaction equilibrium is not shifted during the separation of free DNA and bound complexes. This presumably occurs because the off-rates under the gel conditions are slow compared to the time of entry of the complexes into the gels (see the Discussion).

Evaluation of Ca^{2+} as an Analogue for Mg^{2+}. Direct assessment of the effect of Mg^{2+} on binding of *Eco*RV to its cognate DNA site is impossible, because the substrate is rapidly cleaved. Ca^{2+} has been shown to inhibit the enzyme and has been used as a Mg^{2+} analogue to examine binding in the presence of metal (*17*, *28*). However, the appropriateness of Ca^{2+} as a Mg^{2+} analogue has been called into question (*19*). To evaluate the function of Ca^{2+} directly, we compared the effects of Ca^{2+} and Mg^{2+} on the binding affinities of four of the substrates (Figure 2). Measurement of K_a values for **UA**, **CI**, **CG**, and **Nsp** in the presence of Mg^{2+} was possible because these substrates were not significantly cleaved in the presence of Mg^{2+} at 22 °C in the 15 min incubation time of the binding reactions. The single-turnover cleavage rates of **UA**, **CI**, and **CG** substrates are 70-, 10^5-, and 10^6-fold lower than that of **TA**, respectively (*28*). The **Nsp** rate was not determined, although our expectation is that no cleavage would be observed.

Association constants measured in the presence of Mg^{2+} vary from 8.3×10^{10} M for **UA** to 1.7×10^8 M for **Nsp** (Table 1). They are consistently 2−4-fold lower than those

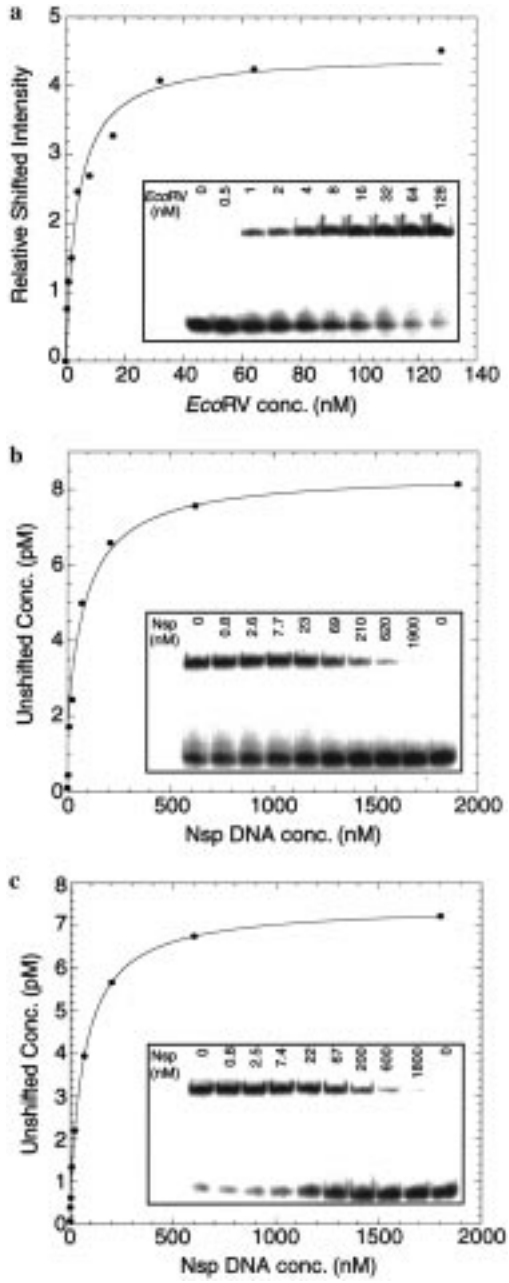

FIGURE 1: Equilibrium gel shift analyses, under different conditions, for the determination of the binding affinity of the nonspecific substrate **Nsp** containing the inverted target site CTATAG. (a) Direct gel shift showing the native gel (inset) and isotherm for the binding reaction of wild-type *Eco*RV in the absence of divalent metal ions. The protein was titrated over the concentration range from 0 to 128 nM, and the concentration of the DNA was 10 pM. The intensity of the shifted DNA band in arbitrary units is on the ordinate. (b) Competition gel shift for the determination of the binding affinity for binding of **Nsp** to the K38A mutant enzyme in the presence of Ca^{2+} ions. The concentration of the protein and of the labeled **CI** substrate DNA were held fixed [an enzyme concentration of 0.2 nM and a **CI** concentration of 8.4 pM, such that approximately 80% of the **CI** DNA is shifted (leftmost lane)]. **Nsp** DNA is then titrated in (lanes 2−9) at concentrations ranging from 0.8 nM to 1.9 μM, at which point nearly all of the **CI** is competed off. The binding isotherm and native gel are shown. The control lane in which enzyme was not present is at the far right. (c) Competition gel shift for the determination of the binding affinity for binding of **Nsp** to the wild-type enzyme in the presence of Mg^{2+} ions. The experiment was performed as described for panel b, but with 7.5 pM labeled **CI** DNA and 1.3 nM *Eco*RV, and with **Nsp** DNA titrated over the range from 0.8 nM to 1.8 μM.

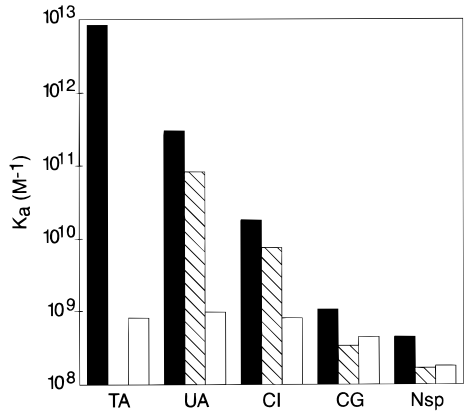

FIGURE 2: Bar graph of values of the thermodynamic association constant K_a for binding of the wild-type *Eco*RV enzyme to the five DNA substrates. Black bars show results of binding reactions performed in the presence of Ca^{2+}, hatched bars in the presence of Mg^{2+}, and white bars in the absence of divalent metal ions. The substrates that were used are indicated along the bottom of the graph. TA denotes the 16-mer duplex substrate 5′-GGGAAA-GA**TA**TCTTGG, where the cognate GATATC site is underlined and the center **TA** step is bold. **UA**, **CI**, and **CG** represent the equivalent center step modifications of **TA**. **Nsp** denotes the substrate 5′-GGGAAACTATAGTTGG, in which the cognate target site is inverted, and is embedded in the same flanking sequence context as the cognate site.

measured in the presence of Ca^{2+}, for each of the four DNA sites (Figure 2). For these DNA base-analogue sites, then, the wide range of binding affinities seen with Ca^{2+} is a near-perfect reflection of the behavior with Mg^{2+}. $\Delta\Delta G°_{bind}$ values, calculated from Ca^{2+} versus Mg^{2+} binding data, are nearly constant at 0.5−0.8 kcal/mol for each of the four substrates **UA**, **CI**, **CG**, and **Nsp**, reflecting the constant small factor by which all affinities are lower in the presence of Mg^{2+} (Table 1). Thus, $\Delta\Delta G°_{bind}$ values among different pairs of these four substrates are nearly identical regardless of which set of binding data are used. This validates the use of Ca^{2+}-dependent binding affinities in correlations with crystal structures of several of the modified complexes (*28*). Two important conclusions immediately follow: (i) Ca^{2+} has the capacity to perform as a superb analogue of Mg^{2+} in binding reactions, and (ii) the active metal Mg^{2+} substantially improves the DNA binding selectivity of *Eco*RV. The performance of these cleavable DNA base-analogue substrates is in sharp contrast to that of the wholly inactive sulfur-containing DNA backbone analogues previously employed to assess the merits of Ca^{2+} as an appropriate substitute for Mg^{2+} (*19*; see the Discussion).

Application to the Study of Enzyme Mutants. The DNA binding affinity of *Eco*RV mutant K38A was examined in reactions with either Ca^{2+} or Mg^{2+} cofactors. Lys38 of each subunit emanates from the amino-terminal end of a long α-helix, and lies in the minor groove of the DNA directly at the center TA step (Figure 4; *33*). The DNA is sharply bent by 50° into the major groove at this position, partially unstacking the TA base pairs and exposing their hydrophobic surfaces in the widened minor groove. The side chain of Lys38 is disordered in most of the *Eco*RV structures which have so far been determined (*11*, *23*, *28*, *33*−*36*), although it has been localized, in several different positions, in recently determined low-temperature structures of ternary uncleaved complexes (N. C. Horton and J. J. Perona, unpublished data;

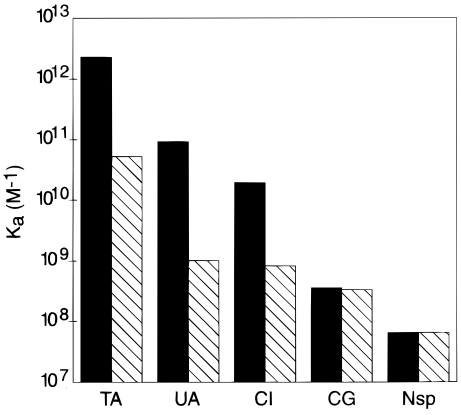

FIGURE 3: Bar graph of values of the thermodynamic association constant K_a for binding of the *Eco*RV K38A mutant enzyme to the five DNA substrates. Black bars show results of binding reactions performed in the presence of Ca^{2+} and hatched bars in the presence of Mg^{2+}. The substrates that were used are as indicated in the legend of Figure 2 and in Materials and Methods.

Figure 4). Lys38 appears not to participate directly in the formation of metal-binding sites, although it does bind to the 5′-phosphate in the enzyme−product DNA−Mg^{2+} complex (*11*). The phosphoryl transfer rate of the K38A mutant is reduced 700-fold compared to that of the wild-type enzyme (M. Sam, S. Lusetti, and J. J. Perona, unpublished data; *37*), sufficiently slow that Mg^{2+} can be used in gel shift studies even with the cognate **TA** substrate.

Comparisons of the binding affinities of *Eco*RV K38A for DNA show that Ca^{2+} and Mg^{2+} have quantitatively different effects. Considerable sequence specificity is present with both metals, but the full range of discrimination corresponds to $\Delta\Delta G°_{bind}$ values of 3.9 kcal/mol for Mg^{2+} and 6.1 kcal/mol for Ca^{2+} (Table 2 and Figure 3). Interestingly, all of the Ca^{2+}-dependent K_a values are very similar between K38A and wild-type *Eco*RV. By this measure, it would thus appear that the ground-state ternary complexes formed by the mutant are identical to those formed by the wild-type enzyme. However, relative to their Ca^{2+}-dependent values, the Mg^{2+}-dependent association constants toward **TA**, **UA**, and **CI** decrease by 25−90-fold for K38A. This is in contrast to the behavior of the wild-type enzyme toward the same base-analogue DNAs, in which strength of binding with Mg^{2+} is only 3-fold weaker than with Ca^{2+} for **UA** and **CI**. It is evident then that the K38A mutant does form a distorted ground-state complex with DNA along the pathway toward the transition state for cleavage, and that detection of this depends on using the catalytically active Mg^{2+} cofactor in binding assays. In the presence of Mg^{2+}, K38A binds 80- and 10-fold weaker to **UA** and **CI**, respectively, than does wild-type *Eco*RV (Tables 1 and 2). Some discrepancies in binding behavior with Mg^{2+} versus Ca^{2+} cofactors were also observed with the weakly active mutant K92A (unpublished results).

DISCUSSION

Binding Specificity in the Absence of Divalent Metal Ions. We have demonstrated by analysis of a set of DNA base-analogue substrates that the binding behavior of *Eco*RV endonuclease differs markedly depending on whether divalent metal ions are present. The effect is evident both in the magnitudes of the binding affinities and in the discrimination among different substrates. At pH 7.5 and 100 mM NaCl, there is 5-fold selectivity between the specific and nonspecific sites in the absence of metal ions, as compared with 10^4-fold selectivity in the presence of Ca^{2+}. The binding affinity for the specific site is enhanced 10^4-fold by Ca^{2+}, from approximately 10^9 to 10^{13} M^{-1}. By contrast, binding affinities for binding to the nonspecific (**Nsp**) and very weakly active **CG** sites are only increased 2.5-fold by Ca^{2+}. Moreover, for these latter sites the affinities without metal ions versus those in the presence of Mg^{2+} are identical within experimental error (Table 1). This is consistent with the notion that divalent metal ions are required for the precise assembly of the enzyme−DNA complex at cognate target sites. At these DNA sequences, the metal ion-binding sites are properly formed and the ternary complexes are on the pathway toward the transition state for phosphoryl transfer. However, at very weakly active and nonspecific sites, metal ions do not improve affinity because the conformations of the binary enzyme−DNA complexes are rearranged (structural adaptability; *2*). This results in disruption of the metal binding sites. Crystal structures of *Eco*RV bound to nonspecific DNA (*33*), and to the **CI** substrate (*28*), provide direct evidence of structural rearrangements at the positions occupied by metal ions in the specific complex.

Our finding that *Eco*RV can manifest at least some DNA binding specificity in the absence of divalent metal ions is consistent with a previous study in which short 22 bp duplex oligodeoxynucleotides were utilized, but the degree of sequence selectivity is considerably smaller in our case. In the earlier work, a difference in affinity of roughly 120-fold at pH 7.5 was reported using both filter binding and gel shift methods (*19*). The solution conditions previously used were 10 mM bis-tris propane and 105 mM NaCl. Affinities for the specific site were similar to ours despite the difference in flanking sequences, but the equilibrium constant for the nonspecific site was found to be only 2.5×10^6 M^{-1}, some 70-fold lower than that determined here (Table 1). Indeed, the K_a of 1.8×10^8 M^{-1} we find for the nonspecific site appears to be the highest yet reported for a nonspecific protein−DNA interaction (*38*). One rationale for the disparate results could be the differences in buffer strength (10 mM bis-tris propane vs 50 mM Tris), which have been shown to significantly affect the K_m values for specific site plasmid cleavage by *Eco*RV (*39*). Additionally, differences in substrate sizes (16-mer vs 22-mer) and flanking sequences (AT-rich with an AAA stretch vs GC-rich) may have significant effects on nonspecific binding affinities in the absence of metal ions.

Other studies carried out under identical conditions of pH, buffer strength, and ionic strength [50 mM Tris (pH 7.5) and 100 mM NaCl] showed an apparent complete lack of binding specificity in the absence of divalent metal ions (*7*). In this work, gel shift analysis of *Eco*RV bound to a series of DNA substrates 55−382 bp in size was performed. No difference was observed in binding affinity regardless of whether the specific GATATC site was present, and multiple shifted bands were observed in each case, indicating binding of the enzyme at diverse sites. However, in this work, the diagnostic gels were run at pH 8.3 in the absence of metal ions. These data have been criticized on the basis of the known strong pH dependence of specific site binding, for

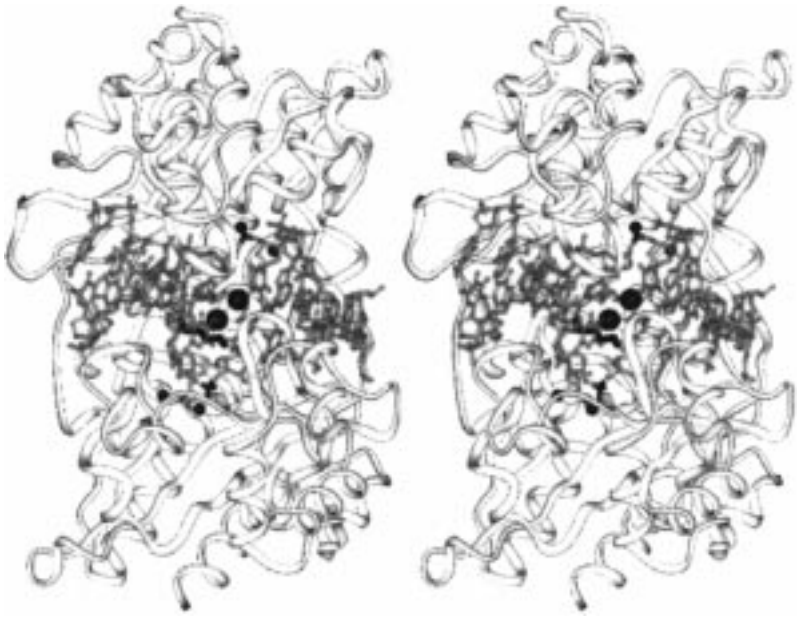

FIGURE 4: Stereoview of the structure of the *Eco*RV−DNA complex. The structure is of the K92A mutant enzyme bound to Mn^{2+} ions determined at 100 K (N. C. Horton and J. J. Perona, unpublished results), in which (unlike previous structures determined at ambient temperatures) the position of the Lys38 side chain can be visualized in one subunit. The scissile phosphates of the DNA are highlighted as dark lines. The position of Lys38 is shown with a dark outline of the side chain; the Lys38 side chain of the other subunit is disordered even at cryocrystallography temperatures. Large spheres denote water molecules binding adjacent to the Lys38 amine group, in the widened DNA minor groove at the center TA step of the GATATC recognition site. Small spheres near the scissile phosphates represent the positions of bound Mn^{2+} ions.

Table 2: Equilibrium Association Constants for Binding of *Eco*RV K38A to Cognate and Base-Analogue Sites

substrate	$K_a(Ca^{2+})$ (M^{-1})	$\Delta\Delta G$-(Ca^{2+}) (kcal/mol)[a]	$K_a(Mg^{2+})$ (M^{-1})	$\Delta\Delta G$-(Mg^{2+}) (kcal/mol)[a]
GA**TA**TC	$(2.3 \pm 0.8) \times 10^{12}$	0.0	$(5.3 \pm 0.6) \times 10^{10}$	0.0
GA**UA**TC	$(9.3 \pm 2.6) \times 10^{10}$	1.9	$(1.0 \pm 0.1) \times 10^{9}$	2.3
GA**CI**TC	$(2.0 \pm 0.3) \times 10^{10}$	2.8	$(8.3 \pm 0.7) \times 10^{8}$	2.4
GA**CG**TC	$(3.6 \pm 0.7) \times 10^{8}$	5.1	$(3.3 \pm 0.6) \times 10^{8}$	3.0
CTATAG (**Nsp**)	$(6.5 \pm 2.0) \times 10^{7}$	6.1	$(6.5 \pm 2.6) \times 10^{7}$	3.9

[a] Free energy differences were calculated from $(K_a)_{WT}/(K_a)_{analogue} = e^{(\Delta\Delta G/RT)}$, with a T of 295 K (*46*).

which extrapolations from measured association constants at lower pH values can be used to predict only 10-fold tighter binding to specific sites at pH 8.3 (*19*). The higher-pH gels may thus destabilize the specific complex to the point where its apparent affinity in experiments performed with large DNA fragments does not significantly differ from that of nonspecific DNA. In our control experiments, we have shown that Ca^{2+}-containing gels do not alter the equilibrium of the complex irrespective of the gel pH, a result consistent with the long half-life of the enzyme−DNA−Ca^{2+} complex [2.8×10^4 s at pH 7.0 (*19*)]. We also find that incubating binding reaction mixtures at pH 7.0 in the absence of metal ions, followed by running on parallel gels at pH 6.0 and 7.0, does not produce a difference in measured K_a values. This is the case even though the half-life of the *Eco*RV-specific DNA complex in the absence of divalent metal ions is only 15−90 s in this pH range, with K_a being 10-fold tighter at pH 6.0 (*19*). Similarly, binding reactions conducted at pH 7.5 in the absence of metals are also not altered by diagnostic gels run at pH 7.0 without metals. We have not, however, re-examined the question of whether gels run at *high* pH in the absence of metals are destabilizing.

Another study in which fluorescence methods were used to measure the binding affinity toward short oligonucleotide duplexes also found no difference in affinity (*20*). The value of K_a determined in this latter work for both specific and nonspecific sites at pH 7.0 was 2×10^7 M^{-1}, 10-fold weaker than observed here for the nonspecific site. However, the fluorescence probes used in this study are relatively insensitive. It is thus unlikely that detection of very small differences in affinity between the specific and nonspecific sites, such as the 5-fold difference determined here, would be possible. The differences in affinity measured toward the respective specific sites are probably reliable, however, and likely arise from differences in the flanking sequence (5′-GAC in ref *16* vs 5′-AAA in this study). A 17-fold variation in binding affinities for binding to the specific site at pH 7.0, depending on flanking sequence, has been reported (*19*).

The varying conditions, methodologies, and choices of substrates used in the different investigations thus render a definitive assessment of the "true" degree of specificity manifested by *Eco*RV in the absence of metal ions elusive. Clearly, each of these variables has significant observed effects. Since both this work and a previous study (*19*) found significant discrimination, however, a proposed division of restriction endonucleases into two fundamentally distinct classes which either do or do not manifest sequence specificity at the binding step seems unwarranted at this stage (*40*). A true molecular basis for this distinction appears to us to be unlikely. Specificity is probably instead partitioned to different degrees at the binding and catalytic steps for all restriction endonucleases (*19*). Indeed, the true ground-state complex is the ternary complex composed of *Eco*RV, DNA, and Mg^{2+}; thus, an assessment of whether specificity occurs at the binding step must be based on a comparison of the binding affinities of *Eco*RV−DNA−Mg^{2+} complexes formed at specifically cleavable versus nonspecific sites. We now

show that a substantial degree of specificity is in fact present, based on the comparison of **UA** and **Nsp** substrates (Table 1; see below). Further, it is also known that the *Eco*RV−DNA binary complex is not on the kinetically preferred pathway toward the transition state for cleavage. This was shown by stopped-flow fluorescence measurements, which revealed that a slow rearrangement step occurs when Mg^{2+} is added to preformed enzyme−DNA complexes (*41*). The evaluation of binding specificity based on measurements in the absence of metal ions, therefore, may be of little importance to the functioning of the enzyme in vivo, where high concentrations of divalent cations are present. Given the similarities in active site structure among all type II restriction endonucleases, and their common requirement for divalent metals, it is likely that this inference is general across the enzyme class.

pH Dependence of Binding. Engler et al. (*19*) found a substantial increase in site selectivity by *Eco*RV at lower pH values. It is interesting to compare the magnitude of the pH effects on specificity with the effects of divalent metal ions reported here. Both lowering of the pH and addition of divalent metal have large effects on specific site binding by *Eco*RV, smaller effects on noncognate site binding, and no effect on nonspecific binding (Table 2). At pH 6.0, where further lowering of pH does not increase the affinity for the specific site, the levels of specific and nonspecific binding differ by 8000-fold (*19*), nearly identical to the effect of 10^4-fold provided by Ca^{2+} [a similar 4500-fold enhancement by Ca^{2+} toward 382 bp restriction fragments has also been reported (*17*)]. Thus, low pH and Ca^{2+} ions have quantitatively similar effects on binding specificity. This suggests that these different conditions likely promote specificity by a similar mechanism. As previously proposed from studies with *Mun*I (*8*, *9*), relief of charge repulsion at the protein−DNA interface can explain both the effects of pH and divalent metal ions on specificity, and this rationale is supported by evidence from crystallographic studies showing divalent metals bound between negatively charged groups on the enzyme and DNA (*11*, *23*). Charge repulsion between these negatively charged groups apparently can be mitigated either by divalent metal ions or by protons.

Enhancement of Binding Specificity by Mg^{2+}. We have demonstrated that it is possible to determine K_a values by gel shift in the presence of Mg^{2+}, for active complexes with cleavage rates up to 1.6% of that of wild-type *Eco*RV toward specific sites (at this level of activity for 15 min incubations, very weak product bands are observed on the gels, but they do not compromise the precision of the measurements as assessed by standard deviations; Table 1). These determinations of the level of Mg^{2+}-dependent DNA binding, for catalytically active complexes, unambiguously demonstrate the ability of this cofactor to enhance binding specificity. For the wild-type enzyme, Mg^{2+} provides nearly 500-fold tighter binding to **UA** compared with that to **Nsp**, whereas in the absence of metal ions the level of discrimination between these substrates is only 5.5-fold (Table 1 and Figure 2). The enhancement of DNA binding specificity provided by Mg^{2+} is given by the ratio of these numbers, and so approaches 100-fold. The binding affinities for the nonspecific site are identical so that all of the improved specificity arises from tighter binding of **UA**.

Prior attempts to assess whether Mg^{2+} could enhance the DNA binding specificity of *Eco*RV have produced inconsistent findings. A set of five hydrolysis-resistant oligodeoxynucleotides, containing phosphate, sugar, or base modifications in the context of the specific site, were not bound specifically in either the presence or absence of Mg^{2+} (*24*). This conclusion was reached from the observation of multiple shifted bands in gel retardation experiments. Measurements of K_a for two of these substrates by filter binding showed very small 2−4-fold enhancements by Mg^{2+} (*19*). It was concluded from this work that Mg^{2+} does not significantly stimulate DNA binding by *Eco*RV. However, our finding of strong Mg^{2+} binding enhancement for active complexes clearly demonstrates that this conclusion drawn from the study of wholly inactive complexes is incorrect. The inactive modified DNAs likely cannot be cleaved because they adopt an aberrant conformation upon enzyme binding, which results in the weakening or elimination of metal binding sites. It is also important to note that, even if significant Mg^{2+} binding stimulation *had* been detected, the data could not be interpreted to mean that Mg^{2+} *enhances specificity*. Assessments of specificity instead require measurements to be made in both the presence and absence of Mg^{2+}, and for both specifically cleavable and nonspecific sites.

Other experiments designed to evaluate whether Mg^{2+} enhances specificity utilized the mutant enzymes D90A and D90A/D74A/E45A, each of which is severely reduced in catalytic activity (*21*, *25*, *42*). Asp90, Asp74, and Glu45 of *Eco*RV are located in the active site and have been observed in cocrystal structures to directly ligate divalent metal ions (*11*, *23*, *28*, *36*). In concordance with similar mutational studies with *Mun*I, the mutants exhibited increased specificities of 10−100-fold in the absence of Mg^{2+}, presumably arising from the relief of electrostatic repulsion between DNA phosphates and protein carboxylates. When Mg^{2+} was added, the affinities for nonspecific DNA decreased and those for specific DNA increased, giving rise to apparently very large enhancements of specificity (*21*, *25*). However, while the overall conclusion is in agreement with that reached here on the basis of the study of active complexes, neither the deduced mechanism nor the magnitude of these Mg^{2+}-dependent enhancements is a reliable indicator of the functioning of the wild-type enzyme. Because the amino acids directly ligating Mg^{2+} were removed, the enhancement of specificity observed in the mutants cannot arise in the same manner as for wild-type *Eco*RV. This is particularly evident for the triple mutant, for which binding stimulation is likely due to nonspecific electrostatic effects (all known Mg^{2+} binding sites are removed in this mutant). By contrast, the active DNA base analogues studied here preserve all the moieties which directly ligate metal ions.

It is not known whether our finding that Mg^{2+} enhances both the binding affinity and the DNA site selectivity of *Eco*RV also holds for other restriction endonucleases. For *Eco*RI, identical values of K_a were obtained in the absence or presence of Mg^{2+}, for several slowly cleaved sites differing from the cognate sequence by one base pair (*43*). This parallels the behavior of *Eco*RV toward the slowly cleaved **CG** site, for which Mg^{2+} similarly does not improve affinity. It is known that *Eco*RI exhibits substantially more sequence selectivity in the absence of metal ions than does *Eco*RV (*3*, *4*). This suggests that Mg^{2+} may not provide as large an

enhancement in binding specificity toward better-cleaved base-analogue sites in this case.

Suitability of Ca^{2+} as an Analogue for Mg^{2+} in Binding Assays. The Ca^{2+}-dependent equilibrium association constants of wild-type *Eco*RV for DNA are consistently 2−4-fold higher than those determined in the presence of Mg^{2+}, for a set of substrates spanning a 600-fold range of binding affinities (**UA**, **CI**, **CG**, and **Nsp**; Table 1). Because the difference in affinities is constant, $\Delta\Delta G°_{bind}$ values among the substrates are nearly identical when calculated with either Ca^{2+} or Mg^{2+} data (Table 1). This definitively establishes that Ca^{2+} has the capacity to perform as a superb analogue for Mg^{2+} in the binding reaction (*28*). The suitability of Ca^{2+} is consistent with the ability of this cation to occupy the same binding sites as Mg^{2+}, as revealed by crystal structures (*11*, *23*, *28*).

Although Ca^{2+} appears to be an optimal analogue for these particular substrates, it nonetheless cannot promote catalysis. The detailed rationale for this is not yet known, but at least three possibilities can be envisioned. First, the ionic radius of Ca^{2+} is larger than that of active metals Mg^{2+}, Mn^{2+}, and Co^{2+} (*32*) so that Ca^{2+} may be sterically excluded from a required binding site (*36*). Second, the pK_a of an inner-sphere water molecule ligated to Ca^{2+} is higher than for any of the active metals. Crystal structures show a divalent metal ion (Mg^{2+}, Mn^{2+}, or Ca^{2+}) directly ligated to a nonesterified oxygen atom of the scissile phosphate group (*11*, *23*, *28*). The less acidic Ca^{2+} is less able to polarize the P−O bond and consequently renders the phosphorus atom less susceptible to attack by the hydroxide ion nucleophile (*44*). Third, the induced-fit conformational changes of the enzyme−DNA complex en route to the transition state may be altered in the presence of Ca^{2+} so that an inactive conformation of the ternary complex is trapped.

We examined the possible role of metal ions in promoting the formation of a productive ground-state ternary complex by measuring the binding affinity of *Eco*RV for DNA in the presence of either Mg^{2+} or Ca^{2+}, in the context of a weakly active enzyme mutant (K38A). Crystal structures of the K38A mutant suggest that the 700-fold diminished phosphoryl transfer rate is (at least in part) due to an inability to form the precise conformation of the ternary ground-state complex (N. C. Horton, S. Lusetti, and J. J. Perona, unpublished results). The measurements of association constants of K38A, for the full series of DNA base-analogue substrates (Table 2 and Figure 3), indeed reveal that the binding affinities for many substrates are dependent on the specific metal ion. It is thus clear that the differences between Mg^{2+} and Ca^{2+} have the capacity to influence not only the catalytic but also the binding step. We suggest that in K38A the metal ion-binding sites are imprecisely formed due to interference with the induced-fit conformational changes. As a consequence, the potential for specific metal ion properties to become manifested may be greater. Mg^{2+} may be more precise in its geometric requirements for positioning of inner-sphere ligands than Ca^{2+}, as reflected in the fact that the coordination numbers for Ca^{2+} in protein crystal structures are sometimes higher and more variable (*45*). Thus, Mg^{2+} is adversely affected by a subtle mispositioning of residues at a metal binding site in the mutant complex, whereas Ca^{2+} can tolerate small differences. This may explain why the full range of discrimination is preserved for Ca^{2+} but not for Mg^{2+} (for Mg^{2+}, the selectivity for **UA** versus **Nsp** is 500-fold for wild-type *Eco*RV and only 15-fold for K38A; by contrast, the selectivity provided by Ca^{2+} does not significantly differ between the two enzymes).

Our measurements thus show that while Ca^{2+} has the *capacity* to perform as a superb Mg^{2+} analogue at the binding step, there also exist certain modified complexes for which there are quantitative differences. However, the conclusion that Ca^{2+} is entirely unsuitable as an Mg^{2+} analogue was reached on the basis of a study of wholly inactive phosphorothiolate and 4′-thiothymidine DNA analogues (*19*). In these experiments, Ca^{2+} stimulated binding by 200−700-fold for both cognate and sulfur-modified substrates, whereas Mg^{2+} gave very little improvement as compared to measurements in the absence of metal ions. In light of the data presented here, it appears that the uncleavable sulfur analogues may produce a more severe effect, but one of the same general kind, as does K38A. Conformations of the binary enzyme−DNA complexes may be more distorted from the true specific complex in the case of the DNA analogues, resulting in a more severe disparity between Mg^{2+} and Ca^{2+} in their respective abilities to enhance binding affinity (and, perhaps, binding *specificity* as well, if the inference of little effect on nonspecific binding is made). Thus, the consideration of a broad array of modifications (DNA base analogues, sulfur-modified backbone analogues, and enzyme mutants) allows for a better appraisal of the extent to which Ca^{2+} and Mg^{2+} behave similarly.

Implications for Protein Engineering Studies. These and previous studies establish that relief of charge repulsion at the enzyme−DNA interface is essential for the expression of significant DNA sequence specificity by *Eco*RV (and likely for other restriction endonucleases). Further, Mg^{2+} must be present during DNA binding for rapid formation of a productive ternary ground-state complex, showing that the binary enzyme−DNA complex is off the preferred kinetic pathway (*41*). Thus, it seems clear that for *Eco*RV systematic binding measurements on modified complexes, as are needed for protein engineering studies, should not be made in the absence of metals at physiological pH values. It is known that measurements of phosphoryl transfer rates can be made for modified complexes reduced by up to 10^6-fold at the cleavage step (*28*). Our finding that binding measurements in the presence of Mg^{2+} can be made for mutants reduced only by 10^2-fold in phosphoryl transfer is salutary, because it indicates that K_a values of modified complexes exhibiting activities across the entire rate regime from 10^{-6} to 10^{-2} of the wild type can be determined for the true active ground-state complex. In the very active range, it is necessary to either substitute Ca^{2+} or, perhaps, to make measurements at reduced pH values. An assessment of the validity of low-pH measurements has not yet been undertaken. However, it appears that Ca^{2+} does provide an accurate analogue for measurements in the range of the wild type to approximately 10^{-4}-fold reduced catalytic rates. In this range (substrates **TA**, **UA**, and **CI**), $\Delta\Delta G°_{bind}$ values obtained in the presence of Ca^{2+} for both the wild type and K38A mutants are accurate reflections of Mg^{2+}-dependent measurements (Tables 1 and 2). It appears that only for complexes which have substantially altered conformations, and thus altered metal binding site geometry, will the properties distinguishing Mg^{2+} and Ca^{2+} come into play. Indeed, measurements of DNA binding

affinities in the presence of each metal ion, for protein engineering studies in which modified complexes are systematically analyzed, may provide a useful probe of whether particular enzyme or DNA functional groups perturb the conformation of the ground state. Such a strategy may also be useful in the further study of other restriction enzymes and (more generally) metal-dependent nucleic acid-modifying enzymes. However, a thorough investigation of the possible options for performing binding measurements should be undertaken in each case.

ACKNOWLEDGMENT

We thank Jonathan Kohn for assistance in enzyme purification, My Sam for performing some control binding reactions, and Victor Bloomfield for communication of results prior to publication.

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BI9905359