DNA Binding Specificity of the *Eco*RV Restriction Endonuclease Is Increased by Mg^{2+} Binding to a Metal Ion Binding Site Distinct from the Catalytic Center of the Enzyme†

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ABSTRACT: In contrast to many other type II restriction endonucleases, *Eco*RV binds specifically to DNA only in the presence Mg^{2+}. According to the co-crystal structure of an *Eco*RV−DNA complex, Mg^{2+} ion(s) bind to the active site of *Eco*RV liganded by Glu^{45}, Asp^{74}, and Asp^{90}. Here we present experimental evidence suggesting that the *Eco*RV−DNA complex also interacts with Mg^{2+} ions at other sites: (i) We have prepared an *Eco*RV triple mutant, in which all acidic amino acids in the catalytic center are replaced by alanine. This mutant is catalytically inactive. It binds nonspecifically to DNA in the absence of Mg^{2+}, whereas it binds specifically to DNA in the presence of Mg^{2+}. This means that Mg^{2+} induces specific DNA binding in this mutant, although all Mg^{2+} ligands in the catalytic center are removed. Therefore, additional interactions between Mg^{2+} and the *Eco*RV−DNA complex probably occur at sites distinct from the catalytic center. (ii) We have measured the specific and nonspecific DNA binding constants of *Eco*RV and of the triple mutant in the presence and absence of Mg^{2+}. Mg^{2+} reduces nonspecific binding by 3−4 orders of magnitude, presumably because Mg^{2+} ions bound to the DNA have to be released upon complex formation. In contrast, the specific binding of the wild-type enzyme and the triple mutant is increased in the presence of Mg^{2+}. This result can only be explained if a Mg^{2+} ion binds to the specific *Eco*RV−DNA complex probably at a site distinct from the catalytic center. (iii) To locate additional metal ion binding sites in the *Eco*RV−DNA complex, we have determined the cleavage rates of several undecadeoxynucleotides which contain single phosphorothioate linkages in the presence of Mg^{2+} and Mn^{2+}. It turned out that an oligodeoxynucleotide in which the first phosphate group within the GpATATC sequence (p^3) is replaced by an R_p phosphorothioate is cleaved by a factor of 50 more readily in the presence of Mn^{2+} than with Mg^{2+}. This result is interpreted to mean that p^3, which is far away from the active site of *Eco*RV, interacts with a Mg^{2+} ion. (iv) We have produced the *Eco*RV Y219C mutant whose DNA cleavage activity compared to wild-type *Eco*RV is reduced by 3 orders of magnitude. It binds nonspecifically to DNA in the absence of Mg^{2+} but not detectably in the presence of Mg^{2+}. Although the distinct role of Tyr^{219} is unclear at present, it must be pointed out that this amino acid is far away from the active site of *Eco*RV but located in close proximity to amino acid residues *vis à vis* p^3. Hence, the behavior of this mutant also supports the conclusion that an important interaction between Mg^{2+} and the *Eco*RV−DNA complex occurs at a site distinct from the catalytic center.

Restriction endonucleases cleave double-stranded DNA in the presence of the essential cofactor Mg^{2+} with high specificity at palindromic recognition sites 4−8 bp in length [for reviews, see Heitman (1993) and Roberts and Halford (1993)]. Many of these enzymes, e.g., *Eco*RI (Modrich & Zabel, 1976), do not require Mg^{2+} for specific DNA binding. This is not the case for the restriction endonuclease *Eco*RV which does not bind specifically to DNA in the absence of Mg^{2+} (Taylor et al., 1991) but does so in the presence of Mg^{2+} as shown for a catalytically inactive mutant of *Eco*RV (Thielking et al., 1992). Thus, for *Eco*RV, Mg^{2+} ions are not only essential for DNA cleavage but also for specific binding. By comparison of the co-crystal structures of *Eco*RI (Rosenberg, 1991) and *Eco*RV (Winkler et al., 1993), the catalytic centers of these enzymes could be identified, because the amino acids of a $PD^{74}...(D^{90}/E)XK^{92}$ motif (numbers refer to the *Eco*RV endonuclease) are positioned similarly relative to the scissile phosphodiester bond in both structures (Thielking et al., 1991). Based on these findings a common catalytic mechanism for *Eco*RI and *Eco*RV was suggested (Jeltsch et al., 1992, 1993a). Recently, also the co-crystal structure of *Pvu*II was solved, which demonstrates that a D...(E/D)XK motif is also there part of the active site (Cheng et al., 1994). The two acidic amino acid residues of the PD...E/DXK motif could function as ligands for the essential Mg^{2+} ion. Indeed, for *Eco*RI it was shown that a Mg^{2+} ion soaked into the co-crystal takes up a position *vis à vis* the scissile phosphodiester bond and next to the two acidic amino acid residues and initiates cleavage of the DNA in the crystal (Rosenberg, 1991). In *Eco*RV, a third acidic

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amino acid residue (Glu^{45}) that has no counterpart in *Eco*RI was suggested to participate in Mg^{2+} binding (Winkler, 1992). In a recent structure analysis of *Eco*RV−DNA crystals grown in the absence of Mg^{2+} and subsequently soaked with Mg^{2+}, two Mg^{2+} ions were found in the active center of one subunit of the enzyme bound by Glu^{45} and Asp^{74} or Asp^{74} and Asp^{90}, respectively (Kostrewa & Winkler, 1995). In this experiment, however, cleavage of the DNA was not observed in the crystal. The function of these acidic amino acid residues had been investigated before by site-directed mutagenesis experiments (Selent et al., 1992). Asp^{74} and Asp^{90} were shown to be indispensable for catalysis of *Eco*RV, because mutants having alanine or asparagine at these positions were catalytically inactive. Glu^{45} turned out to be less important, because the DNA cleavage activity of the E45Q mutant was only reduced 300-fold, and an E45A mutant was still able to cleave DNA. Interestingly, the catalytically inactive *Eco*RV mutants D90A and D90A/D74A are able to bind DNA specifically in the presence of Mg^{2+} (Thielking et al., 1992; Köhler et al., 1994). Therefore, on one hand these mutants are unable to cleave DNA, presumably because of a deficiency in Mg^{2+} binding, but on the other hand Mg^{2+} enhances the DNA binding specificity of the mutants similarly as of wild-type *Eco*RV by several orders of magnitude. This suggests that either Glu^{45} that was not replaced in the D90A and D74A/D90A mutants is sufficient to support Mg^{2+} binding and, thereby, specific DNA binding or, alternatively, that Mg^{2+} ion(s) other than the catalytic one(s) contribute to specific DNA binding of *Eco*RV. Here we have investigated the contribution of Mg^{2+} to the specificity of *Eco*RV employing site specific mutants of *Eco*RV and chemically modified oligodeoxynucleotide substrates. We will present evidence that one or more additional Mg^{2+} interaction site(s) distinct from the catalytic center must exist and that it (or they) is very important for specific DNA binding of *Eco*RV.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Purification of EcoRV Variants. Site-directed mutagenesis of *Eco*RV was carried out following the method described by Ito et al. (1991). This technique is based on the incorporation of a desired mutation with a PCR primer by two successive PCR reactions. Multiple mutations were introduced sequentially by using a mutated plasmid DNA as template. The presence of the desired mutations as well as the absence of unwanted mutations elsewhere in the gene were confirmed by sequencing both strands of the entire *Eco*RV gene of each mutant. All mutant proteins had an affinity tag of six His residues on their N-terminus to facilitate purification (Wenz et al., 1994). The expression of the *Eco*RV gene which is under the control of a p_{tac} promotor was performed in *Escherichia coli* strain LK111(λ), bearing the plasmid pLBM4422 (Thielking et al., 1991) that harbors the gene for the *Eco*RV-methyl transferase. Protein expression and purification was carried out essentially as described by Wenz et al. (1994).

Oligodeoxynucleotides. Oligodeoxynucleotides were synthesized on solid support with a Milligen cyclone plus DNA synthesizer using β-cyanoethylphosphoramidites and "aminolinker" (AL) containing β-cyanoethylphosphoramidites obtained from Milligen. Two oligodeoxynucleotides were used as PCR primers, namely,

oligoA: TAAGAAACCATTATTATCATGACA

oligoB: CTTGGATTCTCACCAATAAAAAACG

OligoA-AL has an identical sequence as oligoA but contains an amino-linker on its 5′ end that was used to covalently attach a fluorescein label.

Phosphorothioate substituted oligodeoxynucleotides were synthesized and purified as described by Thorogood and Connolly (manuscript in preparation). They have the sequence pGpAp^1Cp^2Gp^3Ap^4Tp^5Ap^6Tp^7Cp^8Gp^9TpC (*Eco*RV recognition site in bold letters) and contain a single phosphorothioate group instead of the phosphate group at positions p^1 to p^9. Eighteen different substrates were obtained (thio-1R, thio-1S, etc.), in which one single nonbridging oxygen atom is replaced by sulfur.

Preparation of Radioactively Labeled 382-mer Shift-DNA. A 382 bp DNA fragment was generated by PCR using a variant of pUHE as template. The fragment contains one centrally located *Eco*RV cleavage site. PCR was carried out in a thermocycler varius V45 (Landgraf, Hannover). Template (1.5 ng) DNA was used in a 100 μL PCR reaction with 0.4 μM of each primer, 0.2 mM dNTPs and 3 μCi of [α-^{32}P]-dATP (Amersham). The reaction was carried out with 5 units of Taq polymerase (Amersham) in Taq 1× reaction buffer (cycle 1, 180 s at 95 °C; cycle 2, 30 s at 54 °C, 60 s at 72 °C, and 30 s at 95 °C; cycle 2 was repeated 35 times; cycle 37, 30 s at 54 °C and 200 s at 72 °C). PCR products were separated by gel electrophoresis on a 6% polyacrylamide gel. Product containing bands were excised and eluted with water. The DNA was concentrated by ultrafiltration with Centricon-30 (Amicon) and purified with a subsequent PCR spin preparation (Qiagen). The concentration of the product was determined spectroscopically using an ϵ^{260nm} coefficient of 5×10^6 mol L^{-1} cm^{-1}.

Gel Electrophoretic Mobility Shift Experiments. ^{32}P labeled 382-mer (5 nM) was incubated with 0−320 nM wild-type *Eco*RV or *Eco*RV mutant in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM βME,[1] 2 mM spermine, 0.1 mg/mL BSA) containing either 1 mM EDTA or 10 mM $MgCl_2$ for 15 min at room temperature. To 10 μL of this mixture, 3 μL of gel loading buffer was added [50% glycerol, 0.25% (w/v) xylene cyanol, 0.15% (w/v) chromotrope FB in binding buffer]. Electrophoresis was carried out in 20 × 20 cm 6% polyacrylamide gels at room temperature either in 0.5× TTE (100 mM Tris, 29 mM taurine, 1.25 mM EDTA) or in 0.5× TT supplemented with 10 mM $MgCl_2$. The detection and quantitation of the radioactive bands was performed using an Instant Imager™ system (Canberra Packard).

Preparation of 382-mer DNA Labeled with Fluorescein. OligoA-AL was labeled with fluorescein isothiocyanate (Sigma) on its "amino-linker" as described by Jeltsch et al. (1993b). Labeling yields were estimated spectroscopically to be 40% using an ϵ^{490nm} coefficient of 7.1×10^4 mol L^{-1} cm^{-1} for the fluorescein label. The labeled product was used as PCR primer under the conditions given above (but without addition of [α-^{32}P]dATP) to amplify a 382-mer PCR product that is labeled on the 5′ end of one strand with fluorescein.

[1] Abbreviations: βME, 2-mercaptoethanol; bp, base pair(s); BSA, bovine serum albumin; K_M, Michaelis−Menten constant; K_{Ass}, association constant; $K_{Ass,sp}$, specific association constant; $K_{Ass,nsp}$, nonspecific association constant; *Sma*-nuc, *Serrratia marcescens* nuclease.

Nonradioactive Footprinting. Fluorescein-labeled 382-mer (100 nM) was incubated with 0.02 unit of DNaseI (USB) or 0.01 unit of *Serratia marcescens* nuclease (kindly provided by P. Friedhoff) in 40 μL of *Eco*RV cleavage buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) for 2 or 10 min at 21 °C. Identical samples were incubated with DNaseI and *S. marcescens* nuclease in the presence of 500 nM catalytically inactive *Eco*RV mutant E45A/D74A/D90A after a 30 min preincubation of the *Eco*RV mutant and the DNA. Subsequently, following the ABI protocol the DNA was precipitated and phenol extracted, and a DNA fragment length standard was added (Genescan 2500 ROX). The sample was loaded in one lane of a 6% polyacrylamide gel containing 7 M urea in an ABI DNA sequencer. Data acquisition and analysis were carried out using the 672 Genescan software package (ABI).

Cleavage of Phosphorothioate Containing Oligodeoxynucleotides. The self-complementary oligodeoxynucleotides containing phosphorothioate substitutions were labeled on their 3′ end using terminal deoxynucleotidyl transferase (USB) and [α-^{32}P]ddATP (Amersham). The labeled compounds were purified by NAP10™ columns (Pharmacia). Cleavage reactions were carried out using 0.1 μM oligodeoxynucleotide and 0.1−200 nM *Eco*RV in 10 mM $MgCl_2$, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5 (Mg-buffer), as well as in 2 mM $MnCl_2$, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5 (Mn-buffer). After appropriate time intervals aliquots were withdrawn, spotted onto DEAE thin layer plates (Macherey & Nagel, Düren) and subjected to homochromatography. The separated spots of products and uncleaved substrates were quantitatively analyzed using an Instant Imager™ (Canberra Packard). The apparent first-order cleavage rate was determined from the initial part of the reaction progress curve.

RESULTS

DNA Binding Specificity of the EcoRV Triple Mutant E45A/D74A/D90A. The *Eco*RV triple mutant E45A/D74A/D90A (EDD triple mutant) was cloned by PCR mutagenesis and purified to homogeneity. In this enzyme variant all amino acids possibly involved in the binding of Mg^{2+} in the catalytic center are replaced by alanine. As expected, the enzyme variant turned out to be catalytically inactive in all standard DNA cleavage tests (data not shown). We have investigated the DNA-binding properties of this variant by gel retardation experiments with a PCR substrate 382 bp in length that contains one *Eco*RV binding site. As shown in Figure 1, in the absence of Mg^{2+} the mutant, like wild-type *Eco*RV, readily forms multiple shifted bands, indicative of nonspecific DNA binding. In the presence of Mg^{2+}, however, the mutant forms only one shifted band, which means that it binds specifically to the *Eco*RV recognition site. This result is surprising, as all amino acid side chains deduced from the crystallographic analysis to be involved in metal ion binding at the active site of *Eco*RV were replaced by a methyl group in the mutant. In order to demonstrate that binding occurs specifically at the *Eco*RV site, we carried out a footprint analysis of the *Eco*RV EDD triple mutant with DNaseI and *S. marcescens* nuclease using the same DNA fragment as employed in the gel shift experiments. This analysis was performed with fluorescently labeled DNA using the ABI DNA sequencer. The *Eco*RV EDD triple mutant turned out to protect 15 phosphate groups (all phosphate groups within the *Eco*RV recognition sequence and five additional ones on both sides of the GATATC sequence) against digestion both by DNaseI and *S. marcescens* nuclease in a cleavage buffer containing 10 mM $MgCl_2$ (Figure 2). This result demonstrates that the *Eco*RV EDD triple mutant specifically binds to the *Eco*RV recognition site.

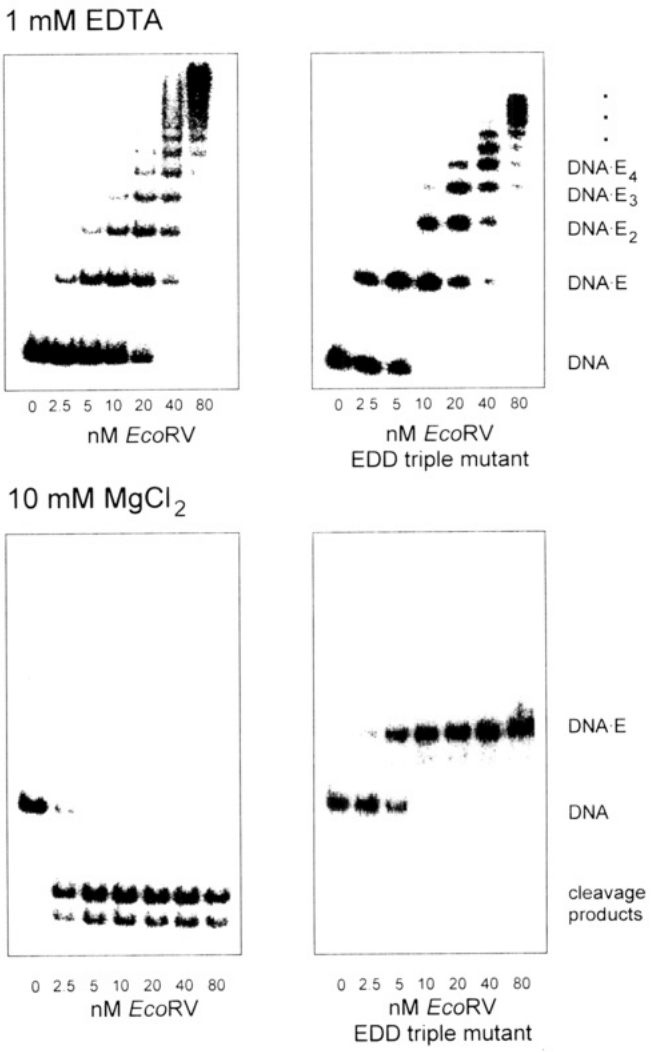

FIGURE 1: Gel electrophoretic mobility shift experiments in the absence (upper panel) and presence of Mg^{2+} (lower panel). Radioactively labeled 382-mer (5 nM) were incubated with wild-type *Eco*RV (left column) or the EDD triple mutant (right column) at various concentrations as indicated.

Conformation of the Specific EcoRV Mutant−DNA Complexes. A comparison of the R_f values of the specifically shifted bands of the two single mutants D90A and D74A, the double mutant D74A/D90A, and the EDD triple mutant shows no difference in the gel mobilities of these protein−DNA complexes (data not shown). This result demonstrates that the bending of the DNA is 44° in all four complexes, as determined for the specific D90A-DNA complex (Stöver et al., 1993). Therefore, the conformations of all four complexes must be very similar to each other.

Quantitative Analysis of the DNA Binding Constants. The results of the gel retardation experiments shown in Figure 1 were further analyzed to determine the DNA binding constants of *Eco*RV and the *Eco*RV EDD triple mutant. Nonspecific DNA binding of the protein is expected to result in several multiple shifted bands in which one, two, three, and so on protein molecules are bound to the DNA. There

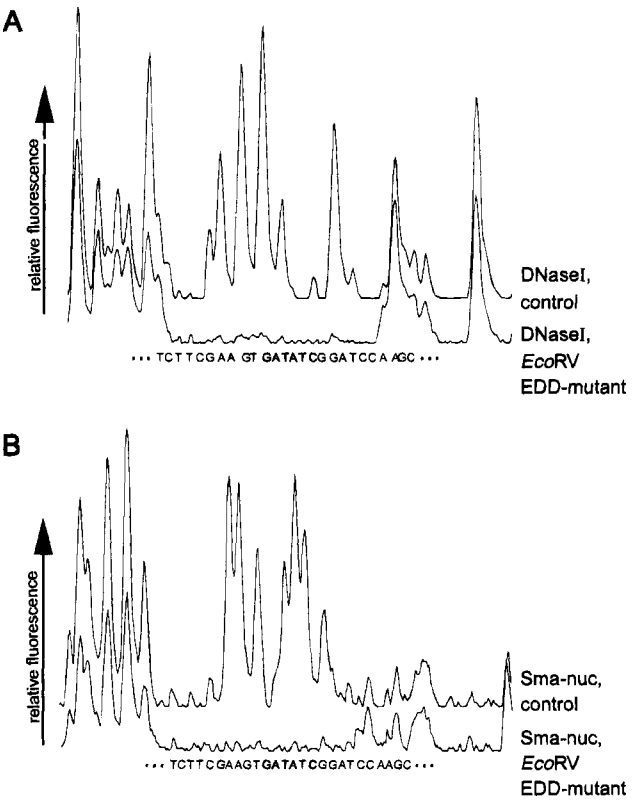

FIGURE 2: Electropherogram showing the relative fluorescence of the DNA fragments resulting from a DNaseI and *S. marcescens* nuclease (*Sma*-nuc) cleavage of the 382-mer PCR product. The 382-mer was cleaved with DNaseI (A) or *Sma*-nuc (B) for 10 min in the absence (upper trace) and in the presence of the *Eco*RV EDD triple mutant (lower trace).

Table 1: DNA Binding Constants of Wild-Type *Eco*RV and the *Eco*RV E45A/D74A/D90A Triple Mutant

	*Eco*RV	E45A/D74A/D90A
1 mM EDTA		
$K_{Ass,sp}$ (M_{DNA}^{-1})[a]	3×10^5–2×10^6	2×10^7
$K_{Ass,nsp}$ (M_{BP}^{-1})[a]	3×10^5–2×10^6	2×10^6
10 mM $MgCl_2$		
$K_{Ass,sp}$ (M_{DNA}^{-1})[a]	$\approx 10^7$	6×10^8
$K_{Ass,nsp}$ (M_{BP}^{-1})[a]	$< 10^2$	5×10^2

[a] The specific binding constants were calculated by assuming that *Eco*RV binds to one specific site. In contrast, nonspecific DNA binding constants were calculated assuming that each single phosphate group defines a nonspecific binding site.

are two ways to determine a non-specific binding constant from this distribution:

(i) The relative intensities of the multiple bands and the free DNA can be used to determine the concentration of bound and free enzyme in each lane. With these data a nonspecific DNA binding constant of *Eco*RV (3×10^5 M^{-1}) and the EDD-triple mutant (2×10^6 M^{-1}) to DNA in the absence of Mg^{2+} was calculated (Table 1). This analysis suffers from one drawback: all complexes that dissociate during electrophoresis will not be included in the analysis. This error can be substantial in cases where much smear is observed. Hence, this analysis will systematically underestimate the binding constant.

(ii) The distribution of the multiple bands can be described by a Poisson distribution if *Eco*RV binds completely nonspecifically to the DNA as suggested by Taylor et al. (1991) for wild-type *Eco*RV. In the case of wild-type *Eco*RV the relative intensities of the multiple shifted bands indeed correspond to a Poisson distribution indicative of an equal affinity of *Eco*RV to the different sites on the DNA-fragment. This distribution is characterized by a μ value that corresponds to the mean number of *Eco*RV molecules bound to each DNA molecule. The probability (P) to find k enzymes bound to one DNA is given by

$$P = \mu^k e^{-\mu}/k!$$

This means that μ can be determined if the relative intensity of only one band is known. Hence, the relative amount of free DNA, which is not affected by the possible dissociation artifact described above, can be used to determine μ, and by using this the concentration of bound and free *Eco*RV molecules and finally the nonspecific binding constant can be calculated. Following this procedure a nonspecific DNA binding constant of 2×10^6 M^{-1} was calculated for wild-type *Eco*RV (Table 1). This value, however, is an upper limit of the binding constant, when a preference for one or more sites exists.

The distribution of multiple shifted bands of the *Eco*RV EDD triple mutant, in contrast to wild-type *Eco*RV, does not correspond to a Poisson distribution. The deviation can be most easily observed in lane 4 (10 nM enzyme) of the gel-shift experiment with the EDD triple mutant in the absence of Mg^{2+} (Figure 1). In this lane, no free DNA is observed but the first shifted band is the most populated one, a relation that is not compatible with completely nonspecific DNA binding (e.g., compare with lane 4 of the corresponding gel shift experiment with wild-type *Eco*RV). Competition experiments with a specific (dCGCGGATATCCGC) and a nonspecific oligodeoxynucleotide (dTATAGAATTCTAT) under these conditions (5 nM 382-mer, 10 nM *Eco*RV EDD triple mutant) confirmed that the EDD triple mutant has a 10-fold preference in binding to the canonical *Eco*RV site over nonspecific DNA, whereas wild-type *Eco*RV does not show a preference for binding to -GATATC- (data not shown). The specific DNA binding constant of *Eco*RV was estimated from K_M values reported for oligodeoxynucleotide substrates [10^{-7} M (Baldwin et al., 1995)] to be approximately 10^7 M^{-1}. Assuming that our preparation contains protein 100% active in binding, the specific DNA binding constant of the EDD triple mutant in the presence of Mg^{2+} can be directly determined from the titration shown in Figure 1 to be 6×10^8 (-2×10^8; $+4 \times 10^8$) M^{-1} (Table 1). If not all proteins are active, the binding constant may be higher, so that the value of 6×10^8 M^{-1} can be taken as an lower limit of the constant. The nonspecific DNA binding constants (Table 1) of wild-type *Eco*RV and the EDD triple mutant in Mg^{2+}-containing buffer were estimated from gel shift experiments at higher protein concentrations. With wild-type *Eco*RV no shift of the cleavage products of the 382-mer was observed up to protein concentrations of 200 nM. With the EDD triple mutant, a second shifted band was observed at concentrations of 160 nM protein (data not shown). Using these data, the nonspecific DNA binding constants of the EDD triple mutant and of wild-type *Eco*RV in the presence of Mg^{2+} can be estimated to be 5×10^2 M^{-1} (EDD-triple mutant) and $< 10^2$ M^{-1} (wild-type *Eco*RV).

DNA Binding of EcoRV Y219C Mutants. During the genetic construction of the mutants, one clone was obtained that contains two amino acid substitutions at the active site

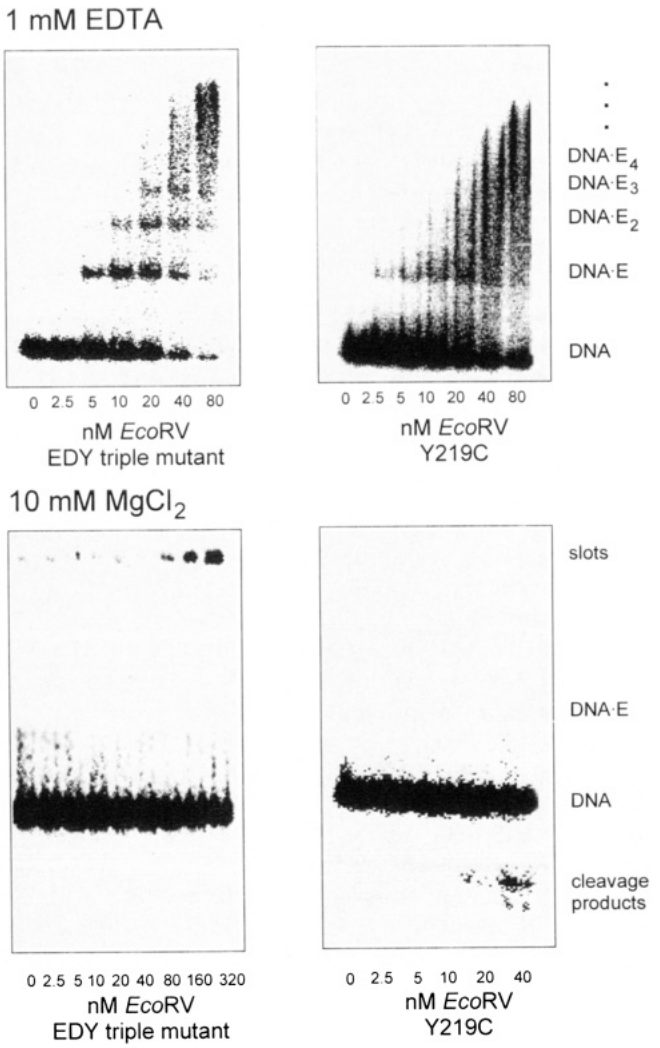

FIGURE 3: Gel electrophoretic mobility shift experiments in the absence (upper panel) and presence of Mg^{2+} (lower panel). Radioactively labeled 382-mer (5 nM) were incubated with the *Eco*RV EDY triple mutant (left) and the *Eco*RV Y219C mutant (right) at various concentrations as indicated.

of the enzyme (E45A/D90A) and one additional mutation (Y219C) that fortuitously occurred in the PCR reaction. As expected, the mutant turned out to be catalytically inactive. Figure 3 shows the DNA binding behavior of this E45A/D90A/Y219C triple mutant (EDY triple mutant). Obviously, the mutant binds to DNA in the absence of Mg^{2+} similarly as wild-type *Eco*RV (nonspecific binding constant determined by the Poisson procedure: 2×10^5 M^{-1}). It does not, however, detectably bind to DNA in the presence of Mg^{2+} ions. Given the conditions of our experiments, it can be estimated that the specific DNA binding constant must be below 10^5 M^{-1}. Thus, this mutant is the first mutant of *Eco*RV described so far that is unable to bind to DNA in the presence of $MgCl_2$. We subsequently confirmed that this is also true for the single mutant Y219C, which is by a factor of 1000 less active than wild-type *Eco*RV and, therefore, can be analyzed in gel-shift experiments in the presence of Mg^{2+}. The nonspecific binding constant determined by the Poisson procedure is 1×10^5 M^{-1}; no DNA binding was detectable in the presence of Mg^{2+} (Figure 3).

Cleavage of Phosphorothioate Containing Oligodeoxynucleotides. We have measured the cleavage rates of pGpAp^{1}Cp^2**G**p^3**A**p^4**T**p^5**A**p^6**T**p^7**C**p^{8}Gp^{9}TpC and all variants thereof in which one of the phosphate groups p^1-p^9 is replaced by an R_p or an S_P phosphorothioate group. In these modified substrates one single nonbridging oxygen atom is replaced by a sulfur atom. The rates of cleavage of these substrates by wild-type *Eco*RV measured in the presence of Mg^{2+} or Mn^{2+} are given in Table 2.

Table 2: Cleavage Rates of Phosphorothioate Substituted Oligodeoxynucleotide Substrates[a]

substrate	$MgCl_2$ rate (k_{ref}/k)[b]	$MnCl_2$ rate (k_{ref}/k)[c]	Mn effect [k_{ref}/k(Mg)/k_{ref}/k(Mn)]
1S	1.1	0.35	3.1
1R	0.24	0.75	0.32
2S	0.51	0.47	1.1
2R	1.3	0.88	1.5
3S	22	2.5	8.8
3R	33	0.65	51
4S	14	11	1.3
4R	24	15	1.6
5S	6.2×10^4	1.2×10^5	0.52
5R	5.2×10^4	2.5×10^4	2.1
6S	$>10^6$	$>10^6$	nd[d]
6R	2.0	1.7	1.2
7S	2.2	9.2	0.24
7R	520	200	2.6
8S	55	82	0.67
8R	27	35	0.77
9S	3.9	1.5	2.6
9R	7.0	15	0.47

[a] All experiments were carried out in duplicate. Errors of the rate constants given are estimated to be ±30%. [b] $k_{ref} = 2.62$ min^{-1}. [c] $k_{ref} = 2.47$ min^{-1}. [d] nd, not determinable.

The rationale behind these experiments was the following: Our results obtained with the EDD triple mutant strongly suggest that at least one additional Mg^{2+} interaction site distinct from the catalytic center exists. Having in mind that this metal ion interaction site is responsible for the specificity of *Eco*RV, it appears reasonable to assume that such a site is located in the *Eco*RV−DNA interface. To locate such an additional site, the relative cleavage rates of phosphorothioates in the presence of Mg^{2+} or Mn^{2+} can be used, because hard metal ions like Mg^{2+} strongly prefer coordination by oxygen whereas Mn^{2+} binds to oxygen and sulfur much more equally (Pecoraro et al., 1984). We therefore decided to look for a phosphorothioate substituted substrate that is cleaved with a reduced rate with Mg^{2+} as cofactor, but whose cleavage rate is rescued by using Mn^{2+} as the divalent metal ion cofactor. The results are compiled in Table 2. Only the Thio-3 substrates show such an effect: Thio-3R is cleaved 33 times more slowly than the unmodified reference substrate in the presence of Mg^{2+}, whereas it is cleaved 1.5 times faster than the unmodified reference substrate in the presence of Mn^{2+}. Therefore, the relative cleavage rate of Thio-3R is 51-fold higher in the presence of Mn^{2+} than in the presence of Mg^{2+}. Similarly, Thio-3S is cleaved 8.8 times more readily in the presence of Mn^{2+} than in the presence of Mg^{2+}. These Mn effects are highly significant, because the Mn-effects at all other phosphate groups uniformly are between 0.24 and 3.1 (average value of thio-1, -2, -4, -5, -6, -7, -8, and -9: 1.36. Standard deviation: 0.92) (Figure 4).

DISCUSSION

It has been the aim of this work to unravel how Mg^{2+} ions increase the DNA binding specificity of *Eco*RV by

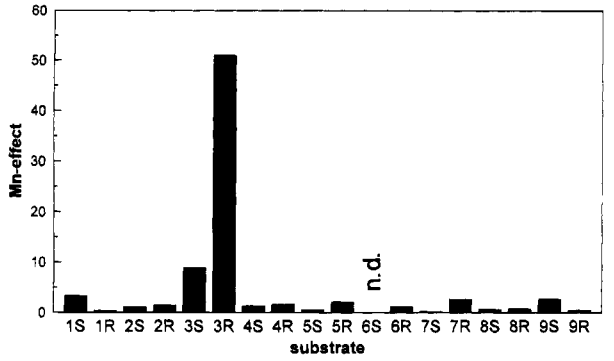

FIGURE 4: Comparison of the Mn effects on the *Eco*RV catalyzed cleavage of 18 undecadeoxynucleotides that contain single phosphorothioate instead of phosphate groups at individual positions within and adjacent to the *Eco*RV recognition sequence (nd, not determinable). The Mn effects are defined as given in Table 2: Mn effect = $[k_{ref}/k(Mg)]/[k_{ref}/k(Mn)]$. They are a quantitative measure of the preference for Mn^{2+} over Mg^{2+} as cofactor in the cleavage of a phosphorothioate substrate by *Eco*RV.

several orders of magnitude. We have prepared an *Eco*RV E45A/D74A/D90A (EDD) triple mutant in which all amino acid side chains are deleted that according to the crystal structure analysis are involved in the binding of the catalytic Mg^{2+} ion(s) (Winkler et al., 1993; Kostrewa & Winkler, 1995). As shown in Figure 1, the DNA binding specificity of the *Eco*RV EDD triple mutant is still strikingly enhanced in binding buffer containing Mg^{2+}, because the protein does no longer form multiple shifted bands in the presence of $MgCl_2$. The data given in Table 1 can be used to further characterize this effect. Obviously, Mg^{2+} induces *two* changes in the DNA binding behavior both of wild-type *Eco*RV and the EDD triple mutant: it reduces the nonspecific DNA binding affinity by 3−4 orders of magnitude, *and* it enhances the specific DNA binding affinity approximately 10−30-fold. This results in an overall increase in specificity by 5 orders of magnitude (Table 1).

In order to understand the involvement of Mg^{2+} ions in the process of DNA binding and cleavage, it appears fruitful to envisage what could happen when *Eco*RV and DNA encounter each other in the presence of Mg^{2+}: given the mM affinity of DNA for Mg^{2+} (Black & Cowan, 1994), it can be assumed that several Mg^{2+} ions are bound to the DNA. It is unclear at present whether the enzyme carries tightly bound Mg^{2+} ions. Some of the DNA bound Mg^{2+} ions are likely to be displaced during complex formation. This need for displacement would reduce the DNA binding constant of *Eco*RV, a mechanism that is well established for monovalent cations (Record et al., 1976). One would not expect *a priori* that mutations in the active site of the enzyme would alter this counterion release effect. Therefore, this scenario nicely explains the observed Mg^{2+} induced reduction of the nonspecific DNA binding constant found with *Eco*RV and the EDD triple mutant.

Not all Mg^{2+} ions, however, have to be removed for specific complex formation. As Mg^{2+} is essential for cleavage, it would be advantageous to use the Mg^{2+} ion sitting close to the scissile phosphodiester bond for catalysis. Furthermore, as Mg^{2+} is essential for specific binding it could well be that Mg^{2+} ions bound somewhere else at the phosphodiester backbone are only reoriented such that they fulfill a bridging function between the DNA and the enzyme. In fact, such a Mg^{2+} binding site has to be postulated to understand our finding that the specific DNA binding affinity is *higher* in the presence of Mg^{2+} than in its absence, because displacement models alone cannot describe such behavior.

What is the contribution of the catalytic Mg^{2+} ion to the specificity of *Eco*RV? One might argue that in the absence of Mg^{2+} electrostatic repulsion between the DNA and the acidic amino acid residues in the catalytic center of *Eco*RV disturbs correct positioning of the DNA. The catalytic Mg^{2+} ion could bridge the negative charges of the protein and the DNA and thereby allow for close recognition contacts. This model would predict that the EDD triple mutant, in which all three negatively charged amino acid residues are deleted, should specifically bind to DNA in the absence of Mg^{2+}. Our data indicate that the mutant indeed binds slightly better to -GATATC- than to other DNA sequences even in the absence of Mg^{2+}; this preference, however, is only 10-fold. We conclude from this result that the compensation of electrostatic repulsion between the DNA and the acidic amino acid residues in the catalytic center of *Eco*RV might contribute a factor of 10 to the DNA binding specificity of the enzyme in the presence of Mg^{2+}. However, the specificity of the EDD triple mutant still is enhanced by an additional factor of 10^5 in a buffer that contains Mg^{2+} ions. We have shown that mutations of Asp74 or Asp90 (Selent et al., 1992), as well as simultaneous mutations of Asp74 and Asp90 (Köhler et al., 1994) or of Glu45, Asp74, and Asp90 (this work) to alanine, completely abolish the catalytic activity of *Eco*RV, indicating that the Mg^{2+} ion essential for catalysis is not properly bound to these *Eco*RV mutant−DNA complexes. The gain in specificity in the presence of Mg^{2+}, however, is qualitatively and quantitatively comparable between wild-type *Eco*RV and all these mutants. One might argue that even in the EDD triple mutant a Mg^{2+} ion could be bound to the active site coordinated by the phosphoryl oxygen of the attacked phosphate group and water molecules bound at the protein−DNA interface. Our data cannot rule out that a Mg^{2+} ion is located there and supports specific DNA binding of the EDD mutant. However, we regard this possibility as unlikely, because Mg^{2+} is bound to the specific DNA complexes of the wild-type enzyme, the D90A mutant (Thielking et al., 1992), the D74A/D90A double mutant (Köhler et al., 1994), and the EDD triple mutant (data not shown) with not too different affinities, a finding which argues against a binding at the catalytic center. A more straightforward alternative explanation for our results could be a second Mg^{2+} ion binding site distinct from the catalytic center in the protein−DNA interface.

To find supporting evidence for and locate additional Mg^{2+} binding sites, we have employed chemically modified DNA substrates: By analyzing the cleavage rates of oligodeoxynucleotides containing a single phosphorothioate group, we have presented experimental evidence that the first phosphate group within the recognition sequence of *Eco*RV (-G**p**ATATC-, p^3) is part of a Mg^{2+} binding site at the *Eco*RV−DNA interface. It was found that an R_P-phosphorothioate at this position was cleaved 30 times more slowly than an unmodified reference substrate. This substrate, however, was cleaved even faster than the reference substrate in the presence of Mn^{2+} resulting in a 50 times higher cleavage rate in the presence of Mn^{2+} than in the presence of Mg^{2+}. Similarly, the S_P-phosphorothioate at position p^3 is cleaved with a 10-fold higher rate in the presence of Mn^{2+} than in the presence of Mg^{2+}. These differences are

significantly higher than the Mn effects observed at all other positions, which were 3.1 at most. Because hard metal ions like Mg^{2+}, unlike Mn^{2+}, do not coordinate to sulfur (Pecoraro et al., 1984), this result suggests that the phosphate group p^3 is involved in a Mg^{2+} interaction site. Replacing the metal coordinating oxygen by sulfur thus prevents cleavage of the substrate in a Mg^{2+} containing buffer, not, however, if Mn^{2+} is present. This approach was first employed by Dahm and Uhlenbeck (1991) to demonstrate a Mg^{2+}–phosphate contact in the hammerhead ribozyme. They found that a hammerhead containing a single phosphorothioate group was cleaved 100-fold more slowly than an unmodified ribozyme in the presence of Mg^{2+} but only 2-fold more slowly in the presence of Mn^{2+}. This effect was interpreted to mean that a metal ion is directly coordinated to the phosphate group in question. This experimental approach to analyze Mg^{2+}–nucleotide interactions has become widely accepted both for ribozymes [Piccirilli et al., 1993; for reviews, see Pyle (1993) and Heidenreich et al. (1993)] and enzymes (Uchiyama et al., 1994). The effect observed with the hammerhead ribozyme (Dahm & Uhlenbeck, 1991) is similar in magnitude to the one we found for the thio-3R substrate with *Eco*RV. Therefore, we conclude that a second metal ion binding site in the *Eco*RV–DNA interface exists near p^3. This interpretation is in excellent agreement with recent crystallographic results: Soaking of Mn^{2+} into an *Eco*RV–DNA co-crystal results in Mn^{2+} binding at two positions, at the active center of the enzyme and near p^3 (F. K. Winkler, personal communicationn). To complement these studies, we have begun to investigate all amino acid residues that according to the crystal structure form contacts to the phosphate groups of the DNA by site-directed mutagenesis (Wenz et al., manuscript in preparation).

In the course of the genetic construction of the active site mutants described in this work, we have found one position that appears to be important for specific DNA binding of *Eco*RV: The *Eco*RV triple mutant E45A/D90A/Y219C (EDY triple mutant) as well as the Y219C single mutant bind to DNA in the absence of Mg^{2+}, albeit with a reduced binding affinity, but do not detectably bind to DNA in the presence of Mg^{2+} ions. Thus, Mg^{2+} reduces the nonspecific *and* specific DNA binding of these mutants. We suggest that the Y219C mutation interferes either with the binding of Mg^{2+} ions to the specific *Eco*RV–DNA complex and/or with an effective release of Mg^{2+} ion(s) during complex formation. As shown in Figure 5, Tyr^{219} is located in the lid of *Eco*RV that enwraps the DNA, far away from the active center of the enzyme. It appears unlikely that an exchange of Tyr^{219} to cysteine has a direct influence on the Mg^{2+} binding at the active center of *Eco*RV, thereby preventing specific DNA binding, whereas three amino acid substitutions at the Mg^{2+} binding site in the catalytic center do not affect the Mg^{2+} induced gain in specificity of the enzyme. Hence, we suggest that the mutation of Tyr^{219} to cysteine has an influence on another Mg^{2+} interaction site, which is more important for specific DNA binding than the catalytic Mg^{2+} binding site. It might be that Tyr^{219} directly is involved in positioning a Mg^{2+} ion at the phosphodiester backbone, as in the co-crystal structure (Kostrewa & Winkler, 1995) the O^{OH}–P distance between Tyr^{219} and the phosphate group (**Ap**AAGATATCTT) is only 5.1 Å. However, there are no other good candidates for Mg^{2+} coordination nearby. It seems more likely, therefore, that Tyr^{219} has an indirect influence on the second Mg^{2+} binding site. Tyr^{219} is part of a hydrophobic cluster that also contains Tyr^{110}. Tyr^{110} on the other hand is located in the loop following β-strand f (Winkler et al., 1993), which also contains Thr^{111}, Ser^{112}, and Lys^{119}, which are all located close to p^3 (Figure 5). Figure 5 also shows that several water molecules are located near p^3 indicative of a hydrophilic surrounding, which can be expected to be favorable for Mg^{2+} binding, too. Interestingly, Thr^{187}, which is part of the recognition loop of *Eco*RV, also closely approaches p^3. Therefore, a direct structural link exists between Tyr^{219}, many amino acids residues that might form contacts to the phosphate groups, p^3, and the recognition loop of *Eco*RV. A mutation of Tyr^{219} to cysteine could disturb the hydrophobic cluster and thereby via Tyr^{110} the conformation of Thr^{111}, Ser^{112}, Lys^{119}, p^3, and Thr^{187}. One has to keep in mind, however, when trying to correlate biochemical results on Mg^{2+} binding to *Eco*RV with the structural data of Kostrewa and Winkler (1995), that no DNA cleavage occurred after soaking the co-crystals with Mg^{2+} ions. This is indicative for the requirement of conformational changes in the complex that are prevented by crystal packing forces.

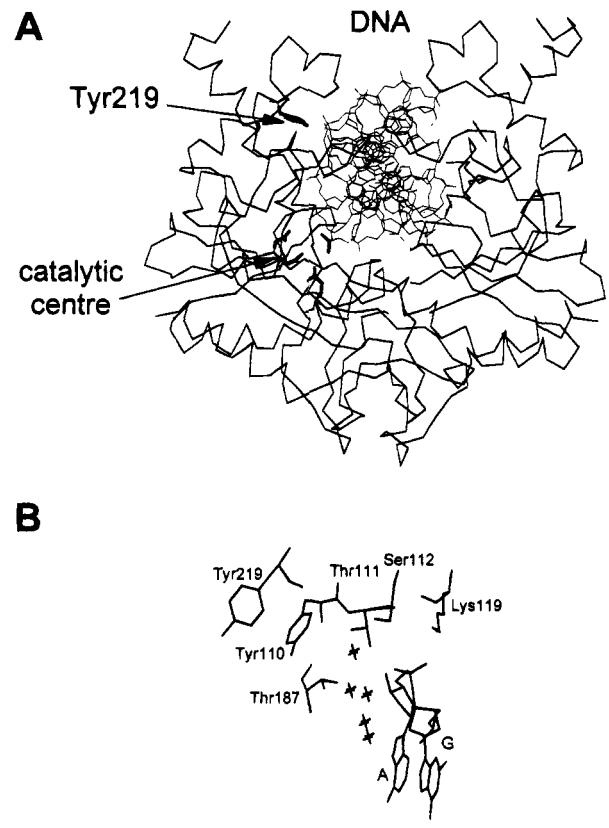

FIGURE 5: (A) Molecular model of the *Eco*RV–DNA complex based on the coordinates of the specific undecamer complex (Kostrewa & Winkler, 1995). The positions of Tyr^{219} and the amino acids in the catalytic center of the enzyme are given in one subunit of the dimeric protein. (B) Structure of the surrounding of the phosphate group p^3 (Gp^3ATATC), which is suggested to be involved in a second Mg^{2+} binding site on the basis of the cleavage experiments with phosphorothioate substituted oligodeoxynucleotides. Thr^{111}, Ser^{112}, Lys^{119}, and Asn^{187} closely approach this phosphate group. Tyr^{110} interacts with Tyr^{219}. There are also several water molecules (*) located in the vicinity of p^3.

In the newly proposed divalent metal ion binding site, the metal ion would be liganded by oxygen atoms (p^3, Ser^{OH}, Thr^{OH}, protein backbone carbonyl groups, water molecules) which are the preferential ligands for hard metal cations like

Mg^{2+} and Ca^{2+}. As there are two negative charges needed to neutralize Mg^{2+} and only one is supplied by the *Eco*RV–DNA complex (p^3), the other must come from the solvent (e.g., OH^-, Cl^-). It should be mentioned here that there are examples of protein structures where Mg^{2+} ions are complexed to nucleotide phosphates but not to an additional negatively charged amino acid: (i) In $p21^{ras}$•GDP Mg^{2+} is bound to GDP-β-phosphate, Ser^{OH}, and four water molecules (Tong et al., 1991). (ii) In the *Eco*RV–DNA product complex a Mg^{2+} ion is bound by the 5′-DNA phosphate, one backbone carbonyl group, and four water molecules (Kostrewa & Winkler, 1995). In both cases, only one negative charge of the phosphate residue is used to partially neutralize the positive charge of the Mg^{2+}, the other negative charge is engaged in an electrostatic interaction with a lysine residue (Wittinghofer et al., 1993; Kostrewa & Winkler, 1995).

In summary, from our studies with the EDD triple mutant we conclude that the specificity of the *Eco*RV restriction endonuclease is enhanced by binding of a Mg^{2+} ion to a site that is distinct from the catalytic center. Moreover, several Mg^{2+} ions must be replaced from the DNA during complex formation, leading to a largely reduced nonspecific DNA binding constant in the presence of Mg^{2+}. The results of the DNA binding experiments obtained with *Eco*RV mutants containing the Y219C amino acid exchange as well as the results of cleavage studies of phosphorothioate substituted substrates in the presence of Mg^{2+} or Mn^{2+} suggest that one additional Mg^{2+} interaction site may be near the first phosphate group within the recognition sequence. This model is in agreement with recent results presented by Vipond et al. (1995). They report complex dependencies of the DNA-cleavage rates by *Eco*RV on the Mg^{2+}, Mn^{2+}, and Ca^{2+} concentration in the cleavage buffer, which could be explained if two metal ion binding sites exist. Vipond et al. (1995) suggested that two metal ions are bound at the active site of each subunit. We would like to point out that it is not the scope of this work to investigate whether there are one or two metal ions bound at the active center of *Eco*RV. The results of Vipond et al. (1995), however, are entirely compatible with binding of the second Mg^{2+} ion to a different binding site, as suggested here on the basis of the results presented above. Perhaps other restriction enzymes that do not bind specifically to DNA in the absence of Mg^{2+} ions, like *Taq*I (Zebala et al., 1992), also contain a second metal binding site at the protein–DNA interface distinct from the active site of the enzyme.

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