Does the Restriction Endonuclease *Eco*RV Employ a Two-Metal-Ion Mechanism for DNA Cleavage?†

Detlef H. Groll, Albert Jeltsch, Ursel Selent, and Alfred Pingoud*

Institut für Biochemie, Fachbereich Biologie, Justus-Liebig-Universität, Giessen, Germany

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ABSTRACT: Two models for the catalytic mechanism of the restriction endonuclease *Eco*RV exist which differ in the number and function of metal ions proposed to be directly involved in catalysis. In one model, two metal ions bound by Glu45, Asp74, and Asp90 are assumed to have a direct catalytic function; in the other, only one metal ion bound by Asp74 and Asp90. We show here that in the presence of Mn^{2+}, the catalytic activity of an *Eco*RV-E45A mutant is only slightly reduced (1.8-fold) as compared to wild type *Eco*RV and that the single-turnover rate constant of DNA cleavage by E45A is reduced only 39-fold, whereas the D74A and D90A mutants are catalytically inactive under all conditions. These findings make an important catalytic function of Glu45, like binding of an essential divalent metal ion, unlikely. In addition, we have analyzed the dependence of the DNA cleavage rate by *Eco*RV and *Eco*RV mutants on the concentration of Mg^{2+} and Mn^{2+}. We found for the wild type enzyme a sigmoidal dependence of the rate of DNA cleavage on the concentration of Mg^{2+} or Mn^{2+}, indicative of at least two metal ions involved in DNA binding and catalysis. This, however, does *not* mean that *Eco*RV follows a two-metal-ion mechanism in DNA cleavage, because also for the E45A mutant a sigmoidal dependence of the rate of DNA cleavage on the Mg^{2+} concentration was found, making metal ion binding to the E45/D74 site unlikely. In contrast, the Y219C mutant shows a hyperbolic dependence. In agreement with results obtained earlier, these findings demonstrate binding of a Mg^{2+} ion at a site influenced by Tyr219, an amino acid residue that is far away from the active site. Metal binding at this site does not have a catalytic role but rather supports specific DNA binding. We conclude that on the basis of our data a two-metal-ion mechanism of DNA cleavage is unlikely for *Eco*RV and that the complex metal ion effects observed are due to metal ion binding at sites that are not directly involved in catalysis.

The type II restriction endonuclease *Eco*RV (Kholmina *et al*., 1980) is a highly specific endonuclease that cleaves double-stranded DNA containing GAT↓ATC sequences as indicated (Schildkraut *et al*., 1984). Like all restriction endonucleases [see reviews by Roberts and Halford (1993), Aggarwal (1995), and Pingoud and Jeltsch (1997)] this enzyme only cleaves DNA in the presence of Mg^{2+} ions or other divalent cations, like Mn^{2+}, Ni^{2+}, or Co^{2+}, but not Ca^{2+}. In the presence of Mn^{2+}, cleavage also occurs at sites differing in one base pair from the canonical site ("star" sites) (Halford *et al*., 1986). Interestingly, distinct from many but like a few other type II restriction enzymes [*Pae*R7 (Ghosh *et al*., 1990), *Taq*I (Zebala *et al*., 1992), *Cfr*9I (Siksnys & Pleckaityte, 1993)], *Eco*RV also needs Mg^{2+} or other divalent cations, e.g. Ca^{2+}, for specific DNA binding (Thielking *et al*., 1992; Vipond & Halford, 1995). This means that divalent metal ions have functional roles in the enzymatic process as well as in the formation of the specificity-determining contacts between the protein and the DNA. Crystallographic studies including metal ion soaking experiments with different divalent cations (Kostrewa & Winkler, 1995) have identified one divalent metal ion binding site formed by Asp74 and Asp90 in the enzyme−DNA complex (Table 1). On the basis of structural and biochemical data as well as a comparison with other restriction endonucleases, there is no reasonable doubt that the Asp74/Asp90 site is essential for DNA cleavage and, hence, that the divalent metal ion bound at this site has a catalytic role (Thielking *et al*., 1991; Winkler, 1992; Selent *et al*., 1992; Jeltsch *et al*., 1992, 1993; Vipond *et al*., 1995; Grabowski *et al*., 1995). However, there exist four observations demanding for at least one additional divalent metal ion binding site in the *Eco*RV−DNA complex: First, in the crystallographic studies with some combinations of divalent metal ions electron density was observed in the catalytic center of *Eco*RV also at a second site formed by Glu45 and Asp74 (Figure 1a and Table 1). Second, metal ion binding at Asp74/Asp90 is not necessary to mediate Mg^{2+} dependent specific DNA binding because the D74A and D90A mutants (Thielking *et al*., 1992), the D74A/D90A double mutant (Köhler *et al*., 1994), as well as the E45A/D74A/D90A triple mutant (Jeltsch *et al*., 1995) all require Mg^{2+} for specific DNA binding. In contrast, Mg^{2+} dependent specific DNA binding is severely disturbed by a mutation at amino acid Tyr219, which is more than 15 Å away from the catalytic center of the enzyme, suggesting that an additional metal ion binding site might be located in the vicinity of Tyr219 (Jeltsch *et al*., 1995). Third, cleavage experiments of phosphorothioate substituted oligonucleotides in the presence of Mg^{2+} or Mn^{2+} gave evidence for a metal ion binding site at the first phosphate within the recognition sequence (G**p**ATATC), which in the complex is near Tyr219 (Jeltsch *et al*., 1995). Fourth, the

* Address correspondence to this author at Institut für Biochemie, Fachbereich Biologie, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany. Phone: +49 641 99 35400. Fax: +49 641 99 35409.

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Table 1: Metal Ion Binding to the Active Sites of the Wild Type *Eco*RV−DNA Complex As Determined from Electron Density Maps Using the Amplitude Differences Observed with and without Metal Ions [Adapted from Kostrewa and Winkler (1995)]

	maximum peak height (multiple of RMS)			
metal ion	D74/D90 (1st subunit)	E45/D74 (1st subunit)	D74/D90 (2nd subunit)	E45/D74 (2nd subunit)
30 mM Mg^{2+}			9.0σ	4.6σ
30 mM Mn^{2+}			14.6σ	
30 mM Co^{2+}	8.4 σ [a]		13.1σ	9.7σ
30 mM Ca^{2+}	8.9σ		11.9σ	
15 mM Mg^{2+}, 15 mM Ca^{2+}	8.9σ		13.3σ	
1 mM Mn^{2+}, 20 mM Ca^{2+}	7.4σ	5.2σ	11.8σ	8.0σ

[a] Only one broad peak is observed.

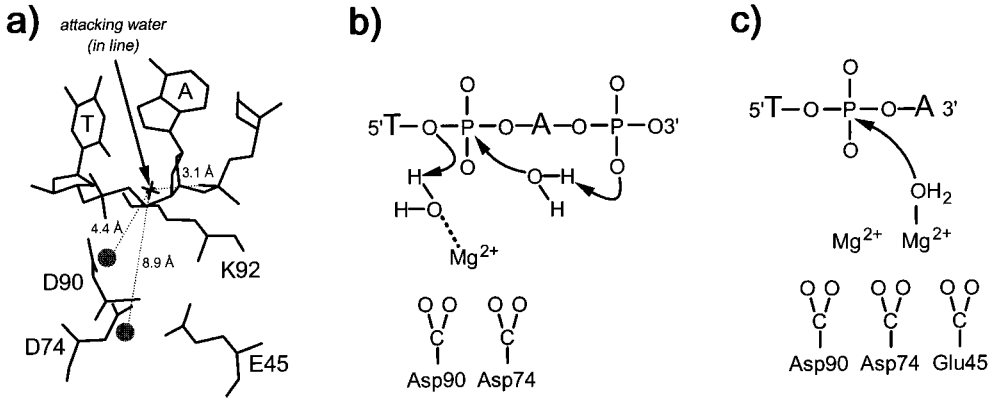

FIGURE 1: (a) Active center of *Eco*RV. The amino acid residues Glu45, Asp74, Asp90, and Lys92, the positions of the Mg^{2+} ions, and the central thymidine and adenine residues within the *Eco*RV recogniton sequence GA*TA*TC are shown (Kostrewa & Winkler, 1995). (b) Schematic drawing of the substrate-assistance model for DNA cleavage by *Eco*RV (Jeltsch *et al*., 1992; 1993). (c) Schematic representation of the two-metal-ion model for DNA cleavage by *Eco*RV (Vipond *et al*., 1995).

dependence of DNA cleavage rates on the concentration of the metal ions when mixtures of Ca^{2+} and Mn^{2+} were used cannot be explained by only one metal ion binding site: In the presence of low Mn^{2+} concentrations, addition of small amounts of Ca^{2+}, which by itself does not support catalysis, stimulates DNA cleavage, whereas larger amounts of Ca^{2+} inhibit catalysis (Vipond *et al*., 1995). On the basis of this "calcium effect" and the crystallographic results mentioned above, a two -metal-ion mechanism was suggested (Vipond *et al*., 1995), which is depicted schematically in Figure 1c. In this model one divalent metal ion bound by Asp74 and Asp90 is responsible for neutralization of the negative charge of the phosphate group to be attacked; the second metal ion bound at the Glu45/Asp74 site serves to activate the attacking water molecule. In a variant of this model the roles of the metal ions are switched (Kostrewa & Winkler, 1995). An alternative model had been proposed before (Jeltsch *et al*., 1992, 1993) in which only one metal ion bound at the Asp74/Asp90 site is assumed to be involved in catalysis (figure 1b). This ion was suggested to be responsible for charge neutralization at the phosphate group and activation of a water molecule for the protonation of the leaving group. The attacking water is proposed to be activated by the *pro*-R_p phosphoryl oxygen of the next phosphate group 3′ to the scissile bond.

It is the aim of this study to investigate the interaction of the *Eco*RV−DNA complex with divalent metal ions by a combination of mutational and kinetic studies. For this purpose, we have determined the dependence of the DNA-cleavage rate of *Eco*RV and *Eco*RV variants on the concentration of divalent metal ions and analyzed these data with respect to the number of bound metal ions.

EXPERIMENTAL PROCEDURES

Cloning, Protein Expression, and Purification. The genes coding for the *Eco*RV mutants E45A, D74A, and D90A (Selent *et al*., 1992) were cloned in pHisRV providing six codons for histidine residues at the 5′ end of the gene. Cloning of the His_6-tagged *Eco*RV mutant Y219C was described in Jeltsch *et al*. (1995). The proteins were expressed and purified by Ni-NTA-chelate affinity chromatography essentially as reported by Wenz *et al*. (1994). A heterodimeric *Eco*RV variant (wt/D90A) which contains a D90A mutation in the catalytic center of only one subunit, carrying a His_6-tag and a GST-tag, was kindly provided by F. Stahl (Wende *et al*., 1996; Stahl *et al*., 1996). After purification, the homogeneous enzyme preparations were dialyzed against storage buffer [30 mM potassium phosphate, pH 7.2, 500 mM NaCl, 0.1 mM DTE, 0.01% (v/v) Lubrol, 70% (v/v) glycerol] supplemented with 0.5 mM EDTA to remove divalent cations. Prior to the oligonucleotide cleavage experiments, all enzyme preparations were extensively dialyzed against storage buffer without EDTA.

λ- and pAT153-DNA Cleavage Experiments. For the λ-DNA cleavage kinetics 2.5 μg of λ-DNA (MBI Fermentas) was incubated at 37 °C with *Eco*RV in 100 μL of cleavage buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/mL BSA) supplemented with 10 mM $MgCl_2$ or 2 mM $MnCl_2$ (0.8 nM DNA, 16 nM *Eco*RV sites). *Eco*RV concentrations were 10 nM (wild type, for experiments in the presence of Mg^{2+}), 0.5 μM (wild type, Mn^{2+}), 1 μM (E45A, Mg^{2+}), 1 μM (E45A, Mn^{2+}), 1 μM (D74A, Mg^{2+} or Mn^{2+}), and 1 μM (D90A, Mg^{2+} or Mn^{2+}). Cleavage of pAT153 plasmid DNA that contains one *Eco*RV site was carried out at ambient temperature using 8 μg of pAT153 in 100 μL of cleavage buffer (30 nM) supplemented

with 10 mM $MgCl_2$ or 0.5 mM $MnCl_2$. *Eco*RV concentrations were 1 nM (wild type, Mg^{2+}), 3 nM (wild type, Mn^{2+}), and 1 μM (E45A, Mg^{2+}), 10 nM (E45A, Mn^{2+}), 1 μM (D74A, Mg^{2+} or Mn^{2+}), and 1 μM (D90A, Mg^{2+} or Mn^{2+}). After appropriate time intervals, aliquots of 10 μL were taken from the reaction mixtures, the reaction was stopped by addition of 50 mM EDTA, and the reaction products were analyzed by agarose gel electrophoresis.

Single-Turnover DNA Cleavage Experiments. Single-turnover kinetics of pAT153 cleavage (5 nM) by wild type *Eco*RV and the E45A mutant (100 nM) were recorded in cleavage buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/mL BSA) supplemented with 2 mM $MnCl_2$ using a SFM3/Q quenched-flow device (Bio-Logic, Claix). Enzyme−DNA mixtures (containing 0.1 mM EDTA to prevent DNA cleavage) were mixed with $MnCl_2$-containing buffer solutions, and after appropriate incubation times (50 ms−5 min) the cleavage reactions were stopped by mixing with an equal volume of 40 mM EDTA. The DNA was precipitated with ethanol and analyzed by agarose gel electrophoresis.

Oligonucleotide Cleavage Experiments. To determine the metal ion dependence of the DNA cleavage rate of *Eco*RV, a self-complementary 20mer oligodeoxynucleotide d(GATC-GACGATATCGTCGATC) was used. The substrate was radioactively labeled on its 5′ end with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (MBI Fermentas). Subsequently, the substrate was desalted by gel filtration on NAP10 columns (Pharmacia) and extensively dialyzed against deionized water. After this procedure the substrate is free of Mg^{2+} ions as indicated by the finding that incubation even with high concentrations of *Eco*RV did not result in DNA cleavage. The absence of metal ions in the labeled oligonucleotide preparations was tested with each individual preparation. To measure the Mg^{2+} dependence of the DNA cleavage rate of *Eco*RV, a premix that contained buffer (final concentrations: 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/mL BSA), oligonucleotide (final concentration: 1 μM), and enzyme was prepared. The reaction was started by addition of divalent metal ions to aliquots of the premix. This procedure ensures that rates can be measured at a very precise and constant enzyme/substrate ratio at different metal ion concentrations. As the cleavage experiments were carried out at substrate saturation, the relative rates measured here are very accurate because pipetting errors regarding substrate concentrations are negligible. After appropriate time intervals, aliquots were withdrawn from the reaction mixture, spotted onto DEAE thin-layer plates (Macherey & Nagel, Düren), and subjected to homochromatography (Brownlee & Sanger, 1969). After chromatography, the spots representing substrate and cleavage products were quantitatively analyzed by area integration using an instant imager (Canberra Packard). The initial DNA cleavage rates at each concentration of the metal ion were determined from the linear part of the reaction progress curve by linear regression of at least four individual points. One typical result is shown in Figure 2. Usually at least three different premixes containing different concentrations of *Eco*RV were required to measure rates at all desired concentrations of the divalent metal ion. All rates at each concentration of metal ions were determined at least in triplicate and averaged. On the basis of the scatter of the results the errors of the rates determined at each metal ion concentration can be estimated to be below ±30%.

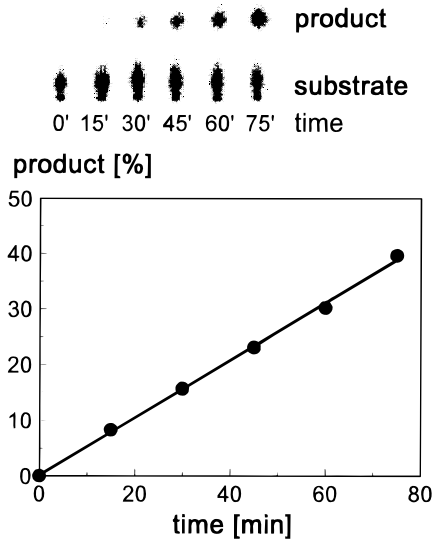

FIGURE 2: Example of the DNA cleavage experiments with *Eco*RV. 1 μM oligonucleotide was cleaved using 0.1 μM *Eco*RV in cleavage buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/mL BSA, and 0.02 mM $MnCl_2$. The substrates and reaction products were separated by homochromatography (upper part) and quantified using an instant imager (lower part).

Analysis of Metal Ion Dependence of Oligonucleotide Cleavage Rates. We have determined the dependence of DNA cleavage rates by *Eco*RV and *Eco*RV mutants on the concentration of Mg^{2+} and Mn^{2+} to find out how many divalent metal ions affect the DNA cleavage rate of the enzyme. To quantitatively analyze the resulting curves with respect to the number and affinity of metal ion binding sites we have used the following approach. Metal ion (Me) binding to a given site i is characterized by:

$$K_{\mathrm{Ass},i} = c_{\mathrm{EM},i}/[c_{\mathrm{Me,free}}\, c_{\mathrm{E},i}]$$

where $c_{\mathrm{EM},i}$ is the concentration of enzymes in which metal ion binding site *i* is occupied, $c_{\mathrm{E},i}$ is the concentration of enzymes in which metal ion binding site *i* is not occupied, $c_{\mathrm{Me,free}}$ is the concentration of free metal ion, and $K_{\mathrm{Ass},i}$ is the binding constant of Me to site *i*.

If the concentration of the metal ions is sufficiently higher than those of the enzyme and the DNA, the free metal ion concentration is always close to the total metal ion concentration c_{Me} ($c_{\mathrm{Me,free}} \approx c_{\mathrm{Me}}$).[1] The probability that site *i* is occupied or free is then given by

[1] This assumption is valid for all experiments with Mg^{2+}. However, some experiments were carried out at concentrations of Mn^{2+} where the metal ion concentration is smaller than the total concentration of nucleotides. We, therefore, have to estimate how metal ion binding to the DNA might affect the concentration of free metal ions in solution. On the basis of published data, on average 3.5 Mn^{2+} ions will bind to the 20mer oligodeoxynucleotide with binding constants of 2000−21 000 M^{-1} (van Steenwinkel *et al*., 1981). If a binding constant of 15 000 M^{-1} is assumed, Mn^{2+} binding to the DNA will not alter the concentration of free metal ions down to 0.1 μM Mn^{2+} by more than 5%. In addition, in the buffer that is used, 100 mM Na^+ is present, which also binds to DNA with a binding constant of 150 M^{-1} (Black & Cowan, 1994). Given the ratios of concentrations of Na^+ and Mn^{2+} (mM *vs* μM = 1000) and of binding constants of the metal ions to DNA (15 000 M^{-1} *vs* 150 M^{-1} = 100), only very few Mn^{2+} ions are expected to bind to the DNA under these condtions. Thus, the assumption that the free concentration of metal ion is similar to the total concentration of metal ion still holds.

$$P_{\text{bound},i} = c_{\text{EM},i}/c_{\text{E,tot}} = c_{\text{Me}}/[c_{\text{Me}} + 1/K_{\text{Ass},i}] \quad (1a)$$

and

$$P_{\text{free},i} = 1 - P_{\text{bound},i} \quad (1b)$$

where $P_{\text{bound},i}$ is the probability that a metal ion is bound at site i, $P_{\text{free},i}$ is the probability that the metal ion binding site i is not occupied, c_{Me} is the total concentration of the metal ion, and $c_{\text{E,tot}}$ is the total concentration of enzyme.

If more than one metal ion binding site is present, two different models must be considered. In one model the metal ions bind independently to the different sites giving rise to 2^n different enzyme−DNA−metal ion species. In this model the probability of each species to occur is given by the product of the appropriate individual probabilities of each binding site to be occupied or not occupied as defined by eq 1a,b. For example, if three sites are present, the probability of a species in which site A and C are occupied, and site B is not occupied is given by

$$P_{\text{A},\neg\text{B,C}} = P_{\text{A}}P_{\neg\text{B}}P_{\text{C}} = c_{\text{Me}}/[c_{\text{Me}} + 1/K_{\text{Ass,A}}]\,\{1 - c_{\text{Me}}/[c_{\text{Me}} + 1/K_{\text{Ass,B}}]\}c_{\text{Me}}/[c_{\text{Me}} + 1/K_{\text{Ass,C}}] \quad (2)$$

Alternatively, binding to n binding sites can occur cooperatively. For simplicity, we have used a Hill model to describe cooperative binding. In this all-or-none model the probability that n metal ions are bound to one enzyme−DNA complex containing n binding sites is given by eq 3 (Cantor & Schimmel, 1980):

$$P_{\text{bound},n} = (c_{\text{Me}})^n/[(c_{\text{Me}})^n + (1/K_{\text{Ass}})^n] \quad (3)$$

For the study presented here, we have used these two models and combinations of them to describe metal ion binding to the *Eco*RV−DNA complex. In the models AB and ABC, respectively, two and three metal ion binding sites are occupied independently. In the model *n*Z, *n* sites are occupied cooperatively. Independent and all-or-none binding of metal ions were combined in the A*n*Z and AB*n*Z models. In both of these models, *n* sites (type Z) are occupied cooperatively and in addition one (A*n*Z) or two (AB*n*Z) sites are occupied independently of each other and of the sites Z. Table 2 summarizes the different models used in this work.

Given the probability of each enzyme−DNA−Me^{2+} species to occur (eq 2), the concentration of each species can be calculated using eq 4:

$$c_i = c_{\text{E,tot}}P_i \quad (4)$$

In the simulations an intrinsic catalytic rate (k_i) is assigned to each species. The theoretical overall rate (k_{theo}) is given by the following equation:

$$k_{\text{theo}} = \sum_i c_i k_i \quad (5)$$

To minimize the number of variables, k_{A}, k_{B}, k_{C}, and $k_{n\text{Z}}$ were set to zero for the fits with the AB, ABC, and AB*n*Z models. This simplification is reasonable, because if two (or more) metal ions are required for cleavage, an enzyme species in which only one binding site is occupied by definition has a negligible catalytic activity. For the fit of the variables to the data, the sum (S) of the squares of the deviations between theoretical rates (k_{theo}) and measured rates (k_{obs}) at each concentration of the metal ions c_{Me} was calculated.

$$S = \sum_{c_{\text{Me}}} [k_{\text{obs}}(c_{\text{Me}}) - k_{\text{theo}}(c_{\text{Me}})]^2 \quad (6)$$

k_{theo} depends on the model, on the intrinsic catalytic rates of each enzyme−DNA−metal ion species (k_i), and on the affinity of each metal ion binding site ($K_{\text{Ass},i}$). By simultaneous variation of the values for all variables, S was minimized using an Excel (Microsoft) worksheet using the "solver" option of Excel. To estimate the range of error of the analyses, we have individually minimized and maximized each variable with variation of all other variables. These simulations were carried out until the fit obtained has an error sum S that is 1.3 times higher than the sum obtained for the optimal fit.

RESULTS

A two-metal-ion mechanism has been put forward for the DNA cleavage reaction of the restriction enzyme *Eco*RV (Vipond *et al*., 1995). This proposal is supported by the observation of a complex dependence of the DNA cleavage rate on the concentrations of Ca^{2+} and Mn^{2+} as well as by the crystallographic finding that under certain conditions two metal ions bind in the catalytic center of *Eco*RV at one site formed by Asp74 and Asp90 and a second one formed by Glu45 and Asp74 (see Table 1). This two-metal-ion mechanism differs from an alternative one suggested earlier, in which only one metal ion has a functional role in catalysis (Jeltsch et al., 1992, 1993). Both models are depicted in Figure 1. The main purpose of this study has been to find out whether *Eco*RV uses a two-metal-ion mechanism for DNA cleavage, i.e., whether Mg^{2+} binding occurs not only at the Asp74/Asp90 site but also at the Glu45/Asp74 site and whether a metal ion bound at the Glu45/Asp74 site has a *catalytic* role. It should be noticed that throughout this paper we will refer to a "two-metal-ion mechanism" as a mechanism in which both metal ions are *directly involved* in the catalytic process of DNA cleavage. We have pursued two experimental strategies: (i) The two-metal-ion mechanism assigns a catalytic role to Glu45. This proposal was tested by analyzing the DNA cleavage properties of Glu45 mutants and comparison with mutants of other catalytic amino acid residues, i.e., Asp74 and Asp90. (ii) We have performed and carefully analyzed detailed kinetic experiments in order to find out how many metal ions are involved in the catalysis of DNA cleavage by *Eco*RV. To this end we have carried out several series of DNA cleavage experiments in the presence of different concentrations of Mg^{2+} or Mn^{2+} with wild type *Eco*RV and three enzyme mutants.

Catalytic Properties of EcoRV Mutants

We have purified the *Eco*RV mutants E45A, D74A, and D90A to homogeneity by Ni-chelate affinity chromatography. Asp74 and Asp90 have been identified to be catalytic residues in the active center of the enzyme (Winkler, 1992; Selent *et al*., 1992; Winkler *et al*., 1993). These amino acid residues are responsible for binding a catalytic Mg^{2+} ion, a similar function as proposed for Glu45 in the two-metal-ion mechanism (Vipond *et al*., 1995). These variants had been analyzed previously using partially purified protein prepara-

Table 2: Different Models Used in This Work To Analyze the Effect of Divalent Metal Ion Binding to the *Eco*RV−DNA Complex on the Rate of DNA Cleavage

model	number of metal ion binding sites	variables[a]
A	one	$K_{Ass,A}$, k_A
AB	two, independent	$K_{Ass,A}$, $K_{Ass,B}$, k_A, k_B, k_{AB}
ABC	three, independent	$K_{Ass,A}$, $K_{Ass,B}$, $K_{Ass,C}$, k_A, k_B, k_C, k_{AB}, k_{BC}, k_{AC}, k_{ABC}
*n*Z	*n* cooperative	$K_{Ass,Z}$, k_{nZ}, *n*
A*n*Z	one independent, *n* cooperative	$K_{Ass,A}$, $K_{Ass,Z}$, k_A, k_{nZ}, k_{AnZ}, *n*
AB*n*Z	two independent, *n* cooperative	$K_{Ass,A}$, $K_{Ass,B}$, $K_{Ass,Z}$, k_A, k_B, k_{nZ}, k_{AB}, k_{BnZ}, k_{AnZ}, k_{ABnZ}, *n*

[a] K_{Ass} is the binding constant, and *k* is the rate of DNA cleavage of each individual *Eco*RV−DNA−Me^{2+} complex, in which different divalent metal ion binding sites are occupied.

Table 3: Relative DNA Cleavage Activities of Wild Type *Eco*RV and Various *Eco*RV Mutants in the Presence of Mg^{2+} or Mn^{2+}

	substrate				
			pAT153		
	λ-DNA		multiple turnover		single turnover
variant	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mn^{2+}
wild type	≡1.0 (520 units/μg)	≡1.0 (1 unit/μg)	≡1.0 (1.7 min^{-1})	≡1.0 (0.21 min^{-1})	≡1.0 (0.24 s^{-1})
E45A	1/5200	1/17	1/120	1/1.8	1/39
D74A	0	0	0	0	0
D90A	0	0	0	0	0

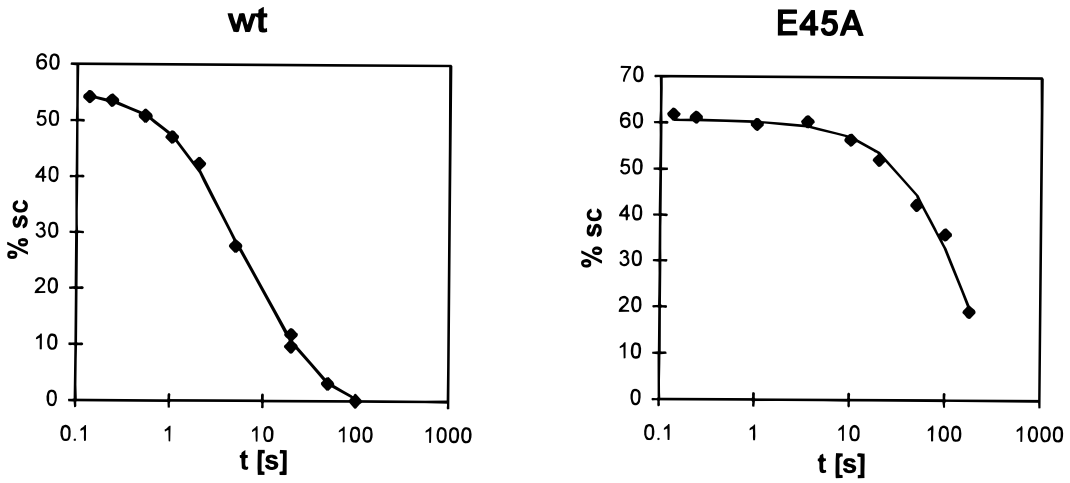

FIGURE 3: Single-turnover experiments of supercoiled (sc) pAT153 cleavage by wild type *Eco*RV and the E45A variant. Experiments were carried out in cleavage buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/mL BSA, and 2 mM $MnCl_2$ with 100 nM enzyme and 5 nM pAT153. The lines represent best fits of the data (wild type, two exponentials with $k_1 = 0.24$ s^{-1} and $k_2 = 0.039$ s^{-1}; E45A, one exponential with $k_1 = 0.0061$ s^{-1}).

tions by Selent *et al*. (1992). The kinetic properties of cleavage of λ-DNA and pAT153 plasmid DNA determined now with the purified mutant proteins are compiled in Table 3. Our results demonstrate that the E45A variant has a reasonable catalytic activity; in the presence of Mn^{2+}, for example, it cleaves the pAT153 substrate only 1.8 times more slowly than wild type *Eco*RV. This result dramatically differs from those obtained with the D74A and D90A mutants, which are catalytically *inactive* under all conditions tested.

As the multiple-turnover rate constants determined so far do not necessarily reflect the rate of the catalytical step *per se*, we have also carried out single-turnover kinetics of pAT153 cleavage by wild type *Eco*RV and E45A in the presence of 2 mM $MnCl_2$. The time course of sc-pAT153 cleavage by wild type *Eco*RV (Figure 3) could be fitted by two exponentials, yielding two rate constants of 0.24 and 0.039 s^{-1}, the faster of which corresponds to the catalytic step, the slower presumably reflecting dissociation, reassociation, and cleavage. Plasmid DNA cleavage by E45A could be fitted by a single exponential with a rate constant of 0.0061 s^{-1} (Figure 3). Thus, the catalytic activity of E45A is reduced 39-fold under single-turnover conditions, whereas the variants D74A and D90A are catalytically inactive. These data demonstrate that the rate of the catalytic step is only slightly reduced in E45A, confirming the interpretation of the multiple-turnover experiments that Glu45 is not a catalytically essential amino acid residue.

Dependence of the Rate of DNA Cleavage by Wild Type EcoRV on the Concentration of Mg^{2+}

In order to find out how many metal ions are involved in DNA cleavage by *Eco*RV, we have analyzed in detail the metal ion dependence of the DNA cleavage rate by *Eco*RV. This approach is based on the fact that a sigmoidal dependence of the cleavage rate on the concentration of Mg^{2+} indicates that more than one metal ion influences the DNA cleavage rate of the enzyme. The degree of sigmoidicity depends on the cooperativity of the sites. It should be emphasized that although sigmoidicity is indicative of the involvement of more than one metal ion, no sigmoidicity does not prove that only one metal ion participates in DNA

Table 4: Quality of the Fit of Measured Data Sets to the Different Models Used To Analyze the Divalent Metal Ion Dependence of Oligonucleotide Cleavage by *Eco*RV and *Eco*RV Mutants

	model	mean residual[a]
wt, Mg^{2+}	A	0.016
	AB	0.0052
	ABC	0.0052
	*n*Z	0.0060
	2Z	0.019
wt/D90A, Mg^{2+}	A	0.021
	AB	0.0095
Y219C, Mg^{2+}	A	0.015
	AB	0.015
wt, Mn^{2+}	ABC	0.027
	AB*n*Z	0.010
	A*n*Z	0.026
E45A, Mn^{2+}	ABC	0.046
	AB*n*Z	0.0084
	A*n*Z	0.037

[a] The quality of the fit is represented by the mean residual, i.e., the mean deviation between all measured values and the corresponding theoretical values.

$$\text{Mean residual} = \sqrt{\sum_{c_{Me}}[k_{obs}(c_{Me}) - k_{theo}(c_{Me})]^2} / \sum_{c_{Me}} k_{obs}(c_{Me})$$

cleavage, because no sigmoidicity would be observed if the affinities of the binding sites differed by orders of magnitude. Thus, absence of sigmoidicity would not exclude the requirement of more than one metal ion involved in catalysis.

On the other hand, it must also be emphasized that, even in the absence of cooperativity in binding, a sigmoidal curve is expected when two or more metal ions are involved in catalysis if the binding constants of both metal ions are similar. Under these conditions the amount of metal ions bound as well as the saturation of each individual binding site depends *hyperbolically* on the concentration of metal ion. However, the DNA cleavage rate is not proportional to the amount of metal ions bound but to the concentration of the enzyme−DNA complex species, in which all necessary sites are occupied, which has a *sigmoidal* dependence on the metal ion concentration. If, for example, two metal ions are involved in catalysis, the following equilibria have to be taken into account:

$$E{-}DNA + 2Me^{2+} \rightleftharpoons E{-}DNA{-}Me^{2+} + Me^{2+} \rightleftharpoons E{-}DNA{-}Me_2^{2+}$$

At low c_{Me} the catalytically inactive E−DNA−Me species will form preferentially. For example, if 10% of all binding sites are occupied, only 1% of the enzyme−substrate complexes are in the catalytically competent E−DNA−Me_2 form. This means that 90% of the metal ion binding under these conditions would not lead to active enzymes. Thus, catalytic activity follows the (hyperbolic) overall saturation of metal ion binding sites in a sigmoidal fashion as described by eq 2.

Any deviation from a hyperbolic shape of the curve should be easily recognized in our experimental setup, because we have accurately determined relative cleavage rates even at very low concentrations of $MgCl_2$. Independent series of cleavage experiments with wild type *Eco*RV were carried out in triplicate at 27 concentrations of $MgCl_2$. In each individual series a sigmoidal dependence of the cleavage rate on the concentration of Mg^{2+} was observed. As shown in Table 4 and Figure 4, these data could be well fitted to a model assuming two metal ion binding sites (model AB, Figure 4a), not, however, if only one Mg^{2+} binding site is assumed (model A, Figure 4b). This result can only be rationalized if at least two metal ions contribute to DNA binding and cleavage by *Eco*RV. In the best fit, both sites have an identical binding constant (Table 5). However, as also indicated in Table 5, both binding constants could be varied to a certain degree. As one would expect, the binding constants turned out to be strongly and inversely correlated: if one constant is raised, a reasonable fit is only obtained if the other one is lowered and *vice versa*.

Inclusion of a third metal ion binding equilibrium (model ABC) does not further improve the fit (Table 4). If in this model a catalytic activity was only assigned to the enzyme−DNA−metal ion complex species in which all three metal ion binding sites are occupied, in the best fit the third binding site has a binding constant high enough to be saturated at all Mg^{2+} concentrations employed. This means that this site does not contribute to the observed rate *vs* Mg^{2+} concentration dependence. We conclude that if more than two sites exist, a third site must have a binding constant in the order of 10^4 M^{-1} or higher.

As more than one Mg^{2+} ion binds to the *Eco*RV−DNA complex, the important question arises of whether these ions interact with the complex cooperatively or independently. Therefore, we also have analyzed whether the data could be fitted by a cooperative binding model (model *n*Z). This analysis resulted in a Hill coefficient of 1.4 but did not result in a better fit (Figure 4c). However, binding of 1.4 metal ions to one *Eco*RV−DNA complex is not meaningful in molecular terms. Hence, we have subsequently analyzed whether the data also could be described assuming that two Mg^{2+} ions cooperatively bind to the *Eco*RV−DNA complex (model *n*Z with $n = 2$). As shown in Figure 4d and Table 4, the best fit obtained with this model is significantly worse than that obtained with the non-cooperative model. We conclude from these results that a non-cooperative model comprising two Mg^{2+} binding sites is best suited to describe the data.

To find out where these Mg^{2+} ions bind, we have carried out similar experiments with three *Eco*RV mutants. Results obtained with a heterodimeric wt/D90A variant and a Y219C mutant are reported in the next paragraphs. The E45A mutant turned out to have too weak a catalytic activity to carry out accurate experiments at low concentrations of Mg^{2+} but a sufficient activity for experiments at low concentration of Mn^{2+} as will be described later.

Mg^{2+} Dependence of the Rate of DNA Cleavage by the EcoRV Heterodimer wt/D90A

*Eco*RV is a homodimeric protein, comprising two catalytic centers which both bind metal ions. Therefore, the possibility had to be tested that the two Mg^{2+} ions found to be required for DNA cleavage by wild type *Eco*RV correspond to one metal ion bound in each catalytic center. This hypothesis could be directly tested by analyzing the Mg^{2+} dependence of the DNA cleavage rate of a heterodimeric *Eco*RV variant that contains a D90A mutation in one catalytic center and, therefore, cannot bind Mg^{2+} at one Asp74/Asp90 site. As shown in Figure 5a, with respect to its Mg^{2+} dependence this variant essentially behaves like the wild type enzyme; in particular, it shows a clear sigmoidal dependence of the

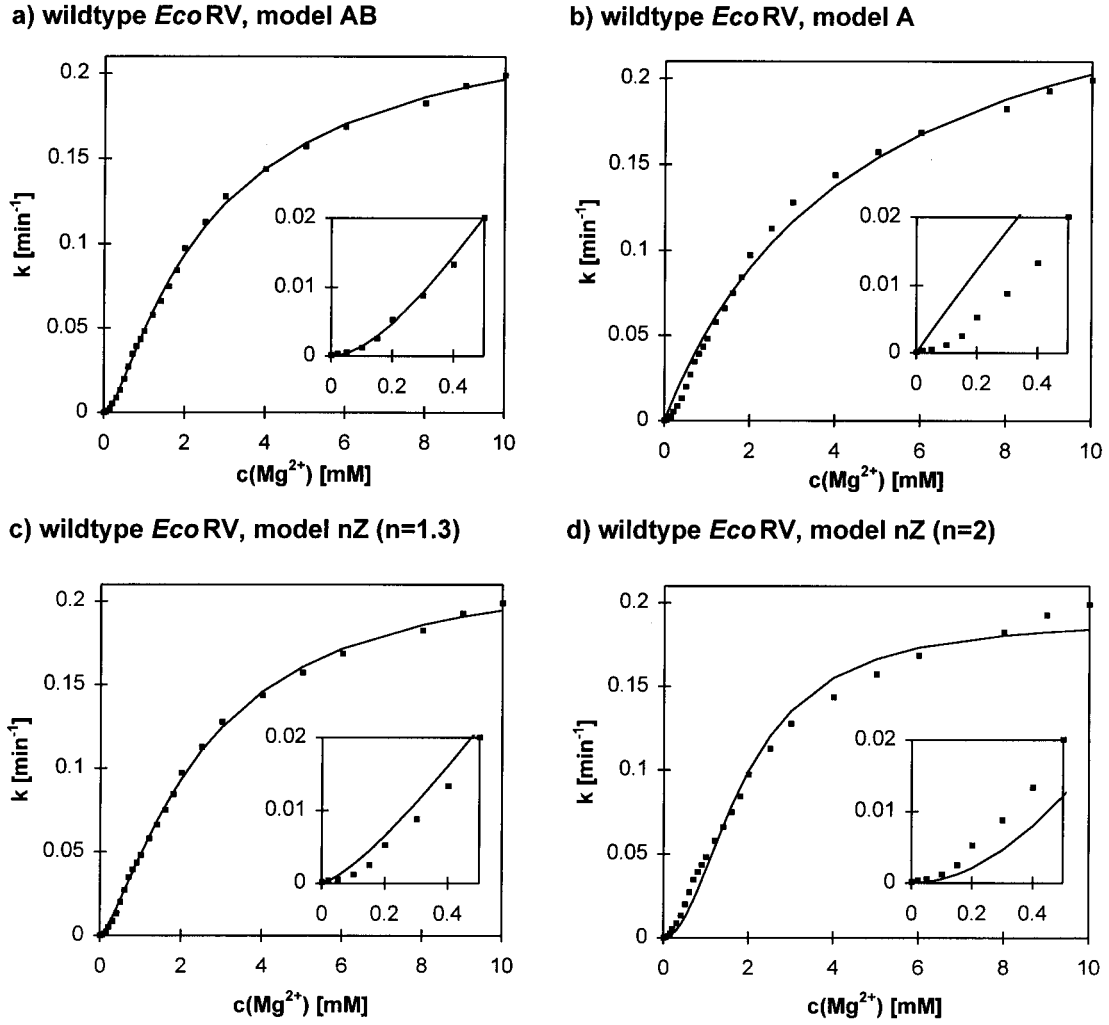

FIGURE 4: Mg^{2+} dependence of the DNA cleavage rate of *Eco*RV. The mean of the measured rates at each concentration is represented by ■. In the inserts a blowup of the initial parts of the curves is shown. (a) Best fit of the data assuming two essential, independent Mg^{2+} binding sites (model AB: $K_{Ass,A} = 790\ M^{-1}$; $K_{Ass,B} = 790\ M^{-1}$; $k_A = 0\ min^{-1}$; $k_B = 0\ min^{-1}$; $k_{AB} = 0.25\ min^{-1}$). (b) Best fit assuming one Mg^{2+} binding site (model A: $K_{Ass,A} = 220\ M^{-1}$; $k_A = 0.30\ min^{-1}$). (c) Best fit using a cooperative Hill model (model *n*Z: $K_{Ass,Z} = 390\ M^{-1}$; $k_{nZ} = 0.22\ min^{-1}$; $n = 1.38$). Note that a cooperative binding of 1.38 metal ions is not meaningful in molecular terms. (d) Best fit assuming cooperative binding of two Mg^{2+} ions (model 2Z: $K_{Ass,Z} = 520\ M^{-1}$; $k_{nZ} = 0.20\ min^{-1}$). The comparison of the plots shows that the non-cooperative model AB is the most plausible model which fits the data.

Table 5: Results of the Analyses of the Divalent Metal Ion Dependence of Oligodeoxynucleotide Cleavage by *Eco*RV and *Eco*RV Mutants

	model	$K_{Ass,A}$ (M^{-1})	$K_{Ass,B}$ (M^{-1})	$K_{Ass,Z}$ (M^{-1})	*n*
wt, Mg^{2+}	AB	790 (360−2550)	790 (360−2550)	na[b]	na
wt/D90A, Mg^{2+}	AB	1190 (580−3340)	1190 (580−3340)	na	na
Y219C, Mg^{2+}	A	630 (490−810)	na	na	na
wt, Mn^{2+}	AB*n*Z	39 000 (3100−49 000)	39 000 (3100−49 000)	38 000 (23 000−74 000)	2.0 (1.65−2.44)
E45A, Mn^{2+}	AB*n*Z	1700 (150−7500)	1400 (150−7500)	1500 (1230−2390)	2.3 (2.0−2.4)

[a] Equilibrium constants are given only for the model yielding the best fit for each data set. Limits of error given in parentheses were estimated as described in Experimental Procedures. [b] Not applicable.

oligonucleotide cleavage rate on the concentration of Mg^{2+}. Again a good fit of the data only could be obtained if two metal ions are assumed to be required for DNA binding and cleavage (Table 4, Figure 5a). Within the limits of error the binding constants obtained are identical to those obtained for wild type *Eco*RV (Table 5). This result demonstrates that more than one Mg^{2+} ion binds to each individual *Eco*RV subunit. Furthermore, it shows that we observe each individual catalytic center in our oligonucleotide cleavage experiments, a finding that is in agreement with our recent result that the two catalytic centers of *Eco*RV are not coupled (Wende *et al.*, 1996; Stahl *et al.*, 1996).

Mg^{2+} Dependence of the Rate of DNA Cleavage by EcoRV Y219C

We had demonstrated previously in DNA binding studies with *Eco*RV that a Mg^{2+} binding site important for specific DNA binding is located near Tyr219 and strongly disturbed by the Tyr219→Cys mutation (Jeltsch *et al.*, 1995). The Y219C mutant has a greatly reduced ability to bind DNA specifically as shown by DNA binding experiments with the catalytically inactive E45A/D90A/Y219C triple mutant in the presence of Mg^{2+} (Jeltsch *et al.*, 1995). It cleaves DNA with a rate reduced by 1−3 orders of magnitude compared to wild type *Eco*RV, depending on the conditions employed.

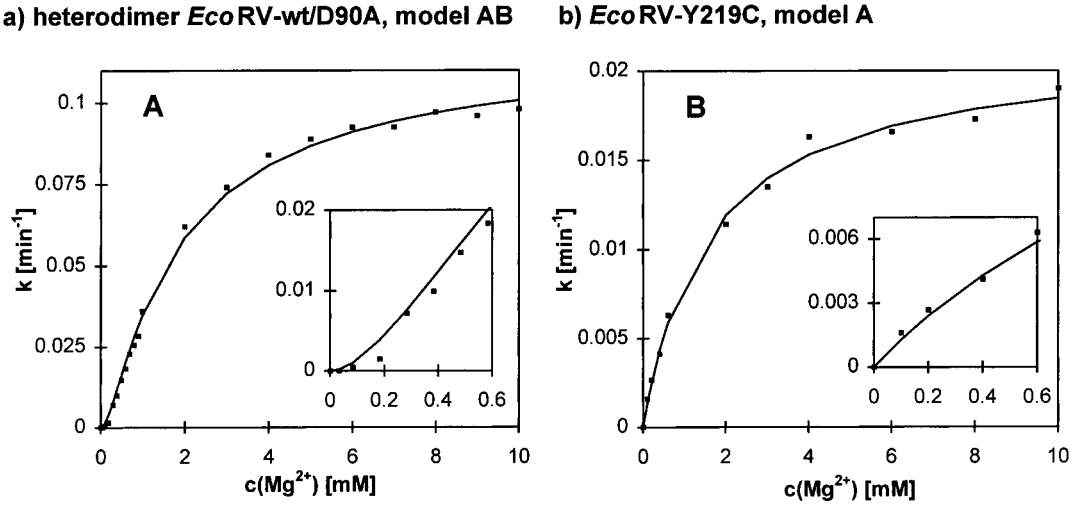

FIGURE 5: Mg^{2+} dependence of the DNA cleavage rate of *Eco*RV mutants. The mean measured rates at each concentration are represented by ■. In the inserts a blowup of the initial part of the curves is shown. (a) Mg^{2+} dependence of the DNA cleavage rate of the heterodimeric *Eco*RV wt/D90A variant. Data were fitted assuming two independent essential Mg^{2+} binding sites (model AB: $K_{Ass,A} = 1190\ M^{-1}$; $K_{Ass,B} = 1190\ M^{-1}$; $k_A = 0\ min^{-1}$; $k_B = 0\ min^{-1}$; $k_{AB} = 0.12\ min^{-1}$). (b) Mg^{2+} dependence of the DNA cleavage rate of the *Eco*RV Y219C mutant. Data were fitted assuming one Mg^{2+} binding site (model A: $K_{Ass,A} = 630\ M^{-1}$; $k_A = 0.021\ min^{-1}$).

Therefore, we have also measured the Mg^{2+} dependence of the DNA cleavage rate of the Y219C mutant. In contrast to wild type *Eco*RV (and the wt/D90A heterodimer), the DNA cleavage rate of the Y219C mutant has a hyperbolic dependence on the Mg^{2+} concentration, which could be readily described by a model assuming only one metal ion binding site (model A, Figure 5b). If the model AB comprising two metal ion binding sites was used, the quality of the fit was not improved (Table 4). In fact, when model AB was used, the best fit of the theoretical curve to the data was obtained when one metal ion binding site was assigned a very high binding constant, such that it is saturated at all Mg^{2+} concentrations. The binding constant for the remaining binding site is similar to that found for each site in wild type *Eco*RV. Obviously, these data can be satisfactorily fitted by assuming that Y219C only needs one Mg^{2+} ion for DNA cleavage. However, this mutant cleaves the oligonucleotide substrate 1 order of magnitude more slowly than wild type *Eco*RV. This result strongly suggests that the second Mg^{2+} binding site, which is responsible for the sigmoidal curve observed with wild type *Eco*RV, is affected by the Y219C mutation. Mg^{2+} binding at this site is not essential for catalysis but enhances the DNA cleavage rate by 1 order of magnitude, presumably by stimulating specific DNA binding (Jeltsch *et al*., 1995).

Given this result, we have reanalyzed the Mg^{2+} dependence of the DNA-cleavage rate of wild type *Eco*RV to find out whether a small residual activity of an *Eco*RV species in which only one metal ion is bound is in agreement with this curve. The analyses showed that a residual activity of up to 1/20 of the activity of the saturated enzyme does not make the fit significantly worse. A good fit of the data was not possible when the residual activity of the one metal ion species was raised to or above 1/10 of the activity of the saturated complex. Thus, the results obtained with wild type *Eco*RV and the Y219C mutant are in agreement with each other.

Dependence of the Rate of DNA Cleavage by Wild Type EcoRV on the Concentration of Mn^{2+}

As mentioned above, Mn^{2+} can replace Mg^{2+} to a certain degree in the DNA cleavage reaction catalyzed by *Eco*RV. However, under these conditions *Eco*RV cleaves DNA less accurately, because cleavage is also observed at sites differing in one base pair from the canonical GATATC sequence (Halford *et al*., 1986). Even more interesting, the "calcium effect" which suggested that more than one metal ion is involved in the DNA cleavage reaction of *Eco*RV, is only observed in a Mn^{2+}-containing buffer (Vipond *et al*., 1995). These observations prompted us to also analyze the Mn^{2+} dependence of wild type *Eco*RV. The Mn^{2+} curve (Figure 6), like the Mg^{2+} curve (Figure 4), clearly shows a sigmoidal shape at low concentrations of the metal ion, implying the existence of more than one Mn^{2+} binding site. However, the concentration dependence shows that Mn^{2+} binds more strongly to the *Eco*RV−DNA complex than Mg^{2+}, a behavior that has been observed before with plasmid DNA substrates (Vermote *et al*., 1992; Vermote & Halford, 1992). On the basis of our cleavage data, Mn^{2+} binding to the *Eco*RV−DNA complex is stronger than Mg^{2+} binding by approximately 1−2 orders of magnitude. Moreover, different from Mg^{2+}, Mn^{2+} decreases the rate of DNA cleavage at concentrations above 0.1 mM. Thus, in addition to the stimulatory sites, one or more inhibitory Mn^{2+} binding sites must exist on the enzyme−substrate complex. A quantitative analysis shows that one inhibitory site (model ABC) is not sufficient to explain the shape of the curve (Figure 6c, Table 4). However, if more than three sites were taken into consideration in the model, too many species would have to be used, because the number of species grows exponentially with the number of binding sites. As in our model, in principle, each species has a certain catalytic activity; too many variables would appear and the results of the analyses would not be meaningful. Therefore, a model allowing for *n* inhibitory sites that are cooperatively occupied was used. This model (AB*n*Z) nicely fits the data if *n* is in the order of 2 (Figure 6a,b and Table 4). A similar fit also might be possible by assuming two or more inhibitory sites that are independent of each other. A satisfactory fit of the data, however, could not be achieved, if only one stimulatory Mn^{2+} binding site was assumed (model A*n*Z, Figure 6d and Table 4). We conclude, therefore, that two stimulatory sites and more than one inhibitory site exist on the enzyme−substrate complex, but whether the inhibitory sites are occupied

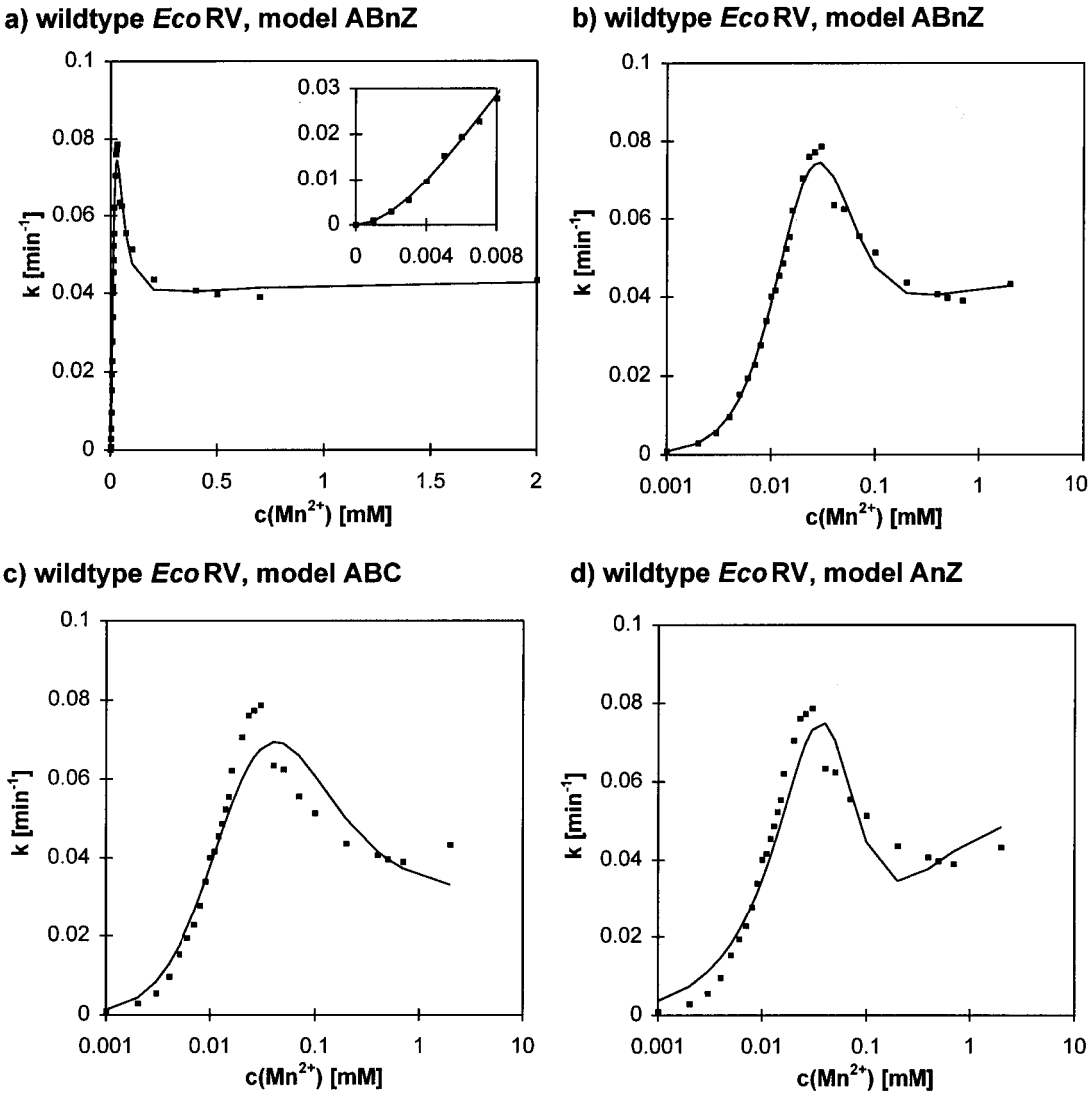

FIGURE 6: Mn^{2+} dependence of the DNA cleavage rate of *Eco*RV. The mean measured rates at each Mn^{2+} concentration are represented by ■. Note that in a, a linear scale on the abscissa is used, whereas for clarity in b−d a logarithmic scale is used; in a and b, the same data are presented on a linear and a half-logarithmic scale, respectively. (a,b) Best fit of the data using the AB*n*Z model ($K_{Ass,A}$ = 39 000 M^{-1}; $K_{Ass,B}$ = 39 000 M^{-1}; $K_{Ass,Z}$ = 38 000 M^{-1}; k_A, k_B, k_{nZ}, k_{AnZ}, k_{BnZ} = 0 min^{-1}; k_{AB} = 0.57 min^{-1}; k_{ABnZ} = 0.044 min^{-1}; n = 2.0). The insert in a is a blowup of the initial part of the curve. (c) Best fit using the ABC model ($K_{Ass,A}$ = 62 000 M^{-1}; $K_{Ass,B}$ = 62 000 M^{-1}; $K_{Ass,C}$ = 62 000 M^{-1}; k_A, k_B, k_C, k_{AC}, k_{BC} = 0 min^{-1}; k_{AB}= 0.40 min^{-1}; k_{ABC} = 0.031 min^{-1}). (d) Best fit using the A*n*Z model ($K_{Ass,A}$ = 5300 M^{-1}; $K_{Ass,Z}$ = 22 000 M^{-1}; k_{nZ} = 0 min^{-1}; k_A= 0.70 min^{-1}; k_{AnZ}= 0.053 min^{-1}; n = 2.5). The comparison of b−d shows that only model AB*n*Z fits the data.

independently or cooperatively cannot be decided by our experiments. It is possible that a similar inhibition of DNA cleavage would be observed with Mg^{2+} at higher concentrations which, however, are not accessible experimentally.

Dependence of the Rate of DNA Cleavage by EcoRV E45A on the Concentration of Mn^{2+}

As the E45A variant is nearly as active as the wild type enzyme in the presence of Mn^{2+}, the Mn^{2+} dependence of the DNA cleavage rate of this mutant could be analyzed and the results compared with those obtained for the wild type enzyme. Qualitatively, the results shown in Figure 7 do not differ from those obtained with wild type *Eco*RV. Again, two stimulatory Mn^{2+} binding sites must be assumed to describe the sigmoidal part of the curve at low concentrations of Mn^{2+} (Table 4). As with wild type *Eco*RV, the occupation of more than one inhibitory site is required to describe the steep descent in activity at higher concentrations of Mn^{2+} (model AB*n*Z, Table 4). At least one of the stimulatory and the inhibitory Mn^{2+} binding sites has a reduced affinity compared to wild type *Eco*RV (Table 5). It should be pointed out that this result differs from that obtained with the Y219C mutant in the presence of Mg^{2+}. Whereas in the latter case one metal ion binding site becomes undetectably weak, in the E45A mutant all sites are still present with moderately reduced affinities toward Mn^{2+}.

DISCUSSION

To elucidate the reaction mechanism of an enzyme implies to understand the transition state between the enzyme−substrate and enzyme−product complex. As the transition state *per definitionem* is not populated, it can only be studied by a combination of kinetic and structural studies. A reasonable mechanism should be structurally plausible and supported by as many biochemical results as possible. The mechanism of an enzymatic reaction can be "proven" only in rare cases but usually one can only accumulate evidence for a certain mechanism and occasionally disprove wrong ones. Currently, two models have been proposed to explain the catalytic mechanism of the restriction enzyme *Eco*RV:

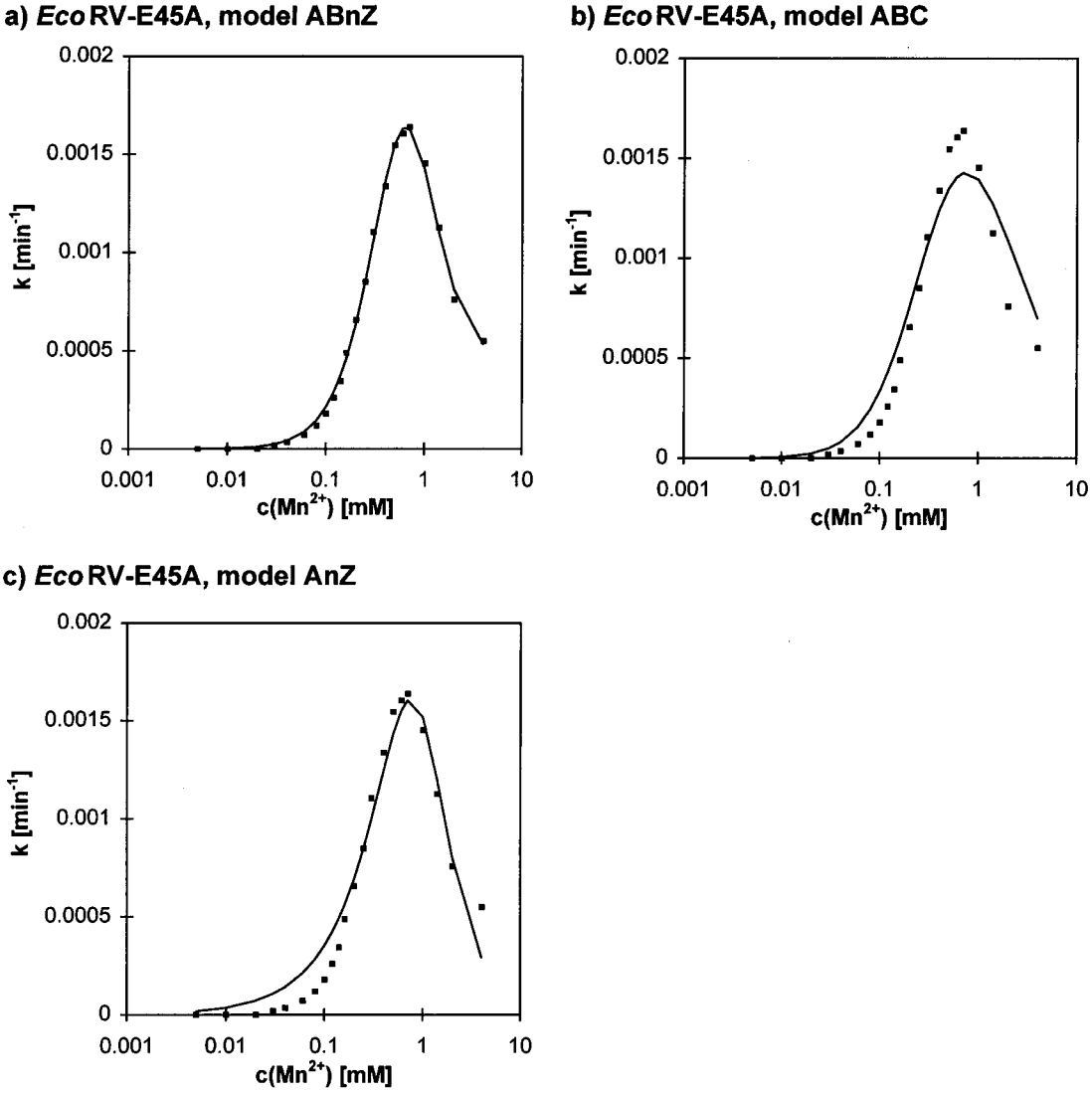

FIGURE 7: Mn^{2+} dependence of the DNA cleavage rate of *Eco*RV E45A. The mean measured rates at each concentration are represented by ■. Note that for clarity a logarithmic scale on the abscissa is used. (a) Best fit of the data using the AB*n*Z model ($K_{Ass,A} = 1700\ M^{-1}$; $K_{Ass,B} = 1400\ M^{-1}$; $K_{Ass,Z} = 1500\ M^{-1}$; k_A, k_B, k_{nZ}, k_{AnZ}, $k_{BnZ} = 0\ min^{-1}$; $k_{AB} = 0.012\ min^{-1}$; $k_{ABnZ} = 5.2 \times 10^{-4}\ min^{-1}$; $n = 2.3$). (b) Best fit using the ABC model ($K_{Ass,A} = 2600\ M^{-1}$; $K_{Ass,B} = 2600\ M^{-1}$; $K_{Ass,C} = 2600\ M^{-1}$; k_A, k_B, k_C, k_{AC}, $k_{BC} = 0\ min^{-1}$; $k_{AB} = 0.010\ min^{-1}$; $k_{ABC} = 0\ min^{-1}$). (c) Best fit using the A*n*Z model ($K_{Ass,A} = 41\ M^{-1}$; $K_{Ass,Z} = 1100\ M^{-1}$; k_{nZ}, $= 0\ min^{-1}$; $k_A = 0.087\ min^{-1}$; $k_{AnZ} = 0\ min^{-1}$; $n = 2.5$). The comparison of the plots shows that, similarly as found for wild type *Eco*RV, only model ABnZ fits the data.

a substrate-assistance model (Figure 1b; Jeltsch *et al.*, 1992, 1993) and a two-metal-ion model (Figure 1c; Vipond *et al.*, 1995). In both mechanisms a Mg^{2+} ion bound by the amino acids Asp74 and Asp90 serves to neutralize the negative charge of the scissile phosphate group. In the substrate-assistance model, the attacking nucleophile is activated by the phosphate group 3′ to the scissile bond and the leaving group is protonated by a water molecule from the hydration sphere of a Mg^{2+} ion bound by Asp74 and Asp90. In the two-metal-ion model, a second metal ion bound at the Glu45/Asp74 site is proposed to be responsible for the activation of the nucleophile. In a variant of the two-metal-ion mechanism the proposed roles of both metal ions are switched (Kostrewa & Winkler, 1995).

In the study presented here we have carried out site-directed mutagenesis experiments as well as detailed kinetic analyses to investigate how many Mg^{2+} ions are involved in DNA cleavage by *Eco*RV. It follows the classical questions of protein−ligand binding analyses pointed out by Scatchard (1949): "How strong? How many? Where? Why?" In this discussion we will first examine results of structural studies in the light of these alternative mechanisms. Subsequently, we will comment on our kinetic data obtained with *Eco*RV mutants in which putative catalytic amino acid residues were replaced and then focus on our investigation of the stoichiometry of metal ion binding to the *Eco*RV−DNA complex.

Is the Two-Metal-Ion Mechanism Structurally Feasible?

Structural studies have shown that one divalent metal ion binds to the active center of *Eco*RV at the Asp74/Asp90 site (Kostrewa & Winkler, 1995). If certain combinations of divalent metal ions are used, electron density is also observed at a second site formed by Glu45 and Asp74, usually in one subunit of the homodimer only (Table 1). However, on the basis of these studies it cannot be decided if the two peaks are a result of simultaneous binding of two metal ions at both sites or of binding of one metal ion at one or the other site averaged over time and/or space. Moreover, the extra density is only weak in most cases. As DNA cleavage does not occur in the crystal after soaking in the metal ion cofactor, it is difficult to estimate whether metal ion binding at each of these sites is necessary or even supportive for catalysis. The structure of the active site as deduced from these studies

is shown in Figure 1a. In this figure the likely position of an attacking water molecule is indicated, which can be derived from the finding that hydrolysis occurs via inversion of configuration at the phosphorus atom (Grasby & Connolly, 1992), suggesting that an in-line attack occurs. Figure 1 also schematically depicts the two mechanisms proposed for *Eco*RV. Obviously, the substrate-assistance model that has been developed on the basis of the structures of *Eco*RI and *Eco*RV is structurally feasible. In the two-metal-ion model proposed by Vipond *et al*. (1995), the metal ion bound at the Glu45/Asp74 site and suggested to be responsible for the activation of the attacking water molecule is approximately 9 Å away from the position of the attacking water, whereas a distance in the order of 2−3 Å would be expected for a hydrogen bond to be formed. Hence, the two-metal-ion model is not in good agreement with the results of the X-ray structure analysis and would require major conformational changes of the enzyme. This is not the case in the Kostrewa and Winkler version of the two-metal-ion mechanism. It should be emphasized that both versions differ from the two-metal-ion mechanism suggested for the 5′-3′ exonucleolytic action of the DNA polymerase I (Beese & Steitz, 1991) according to which the two metal ions are liganded in a symmetrical fashion to the two axial oxygen atoms of the pentacovalent phosphorus atom in the trigonal-bipyramidal transition state.

Is Glu45 a Catalytic Amino Acid Residue?

The two-metal-ion mechanism assigns an important catalytic function to Glu45, namely to bind and position the metal ion responsible for the activation of the nucleophile. It is illuminating to compare results of mutagenesis studies for Glu45 with those obtained for Asp74 and Asp90. The latter amino acid residues chelate the metal ion whose catalytic function is commonly accepted. Whereas the results of mutagenesis experiments at the positions Asp74 and Asp90 are in good agreement with a catalytic role being played by these residues because mutant enzymes are catalytically inactive under all conditions tested, this is not true for Glu45. In fact, in the presence of Mn^{2+} the pAT153 cleavage rate by *Eco*RV E45A is only 1.8-fold reduced as compared to the rate of wild type *Eco*RV. This difference is within the variance expected for different preparations of the same protein. Moreover, in the presence of Mn^{2+} the single-turnover rate constant for DNA cleavage by E45A, which allows direct conclusions to be drawn as to the rate of the chemical step of catalysis, is reduced by only a factor of 40. These data show that Glu45 is dispensable for catalysis under these conditions—a result highly untypical for a catalytically important amino acid residue.

The conclusion that Glu45 is not essential for catalysis is supported by the structural analysis of *Pvu*II, a restriction enzyme that shows strong structural similarity to *Eco*RV (Athanasiadis *et al*., 1994; Cheng *et al*., 1994). In this restriction enzyme a leucine residue (Leu39) is structurally equivalent to Glu45 in *Eco*RV (Athanasiadis *et al*., 1994), again suggesting that Glu45 could not be a catalytic residue.

Could It Be That a Catalytically Important Metal Ion Binds to the E45A Mutant at the Former E45/D74 Site?

The theoretical possibilities exist that a metal ion might bind to the E45A mutant protein at a position equivalent to the E45/D74 site in wild type *Eco*RV and that this ion might still support catalysis. Such an interaction could only be definitely ruled out by metal soaking experiments carried out with this variant. However, binding of a metal ion at this site is unlikely for several reasons: First, in the E45A mutant three negatively charged entities (the phosphate group of the scissile phosphodiester bond and the carboxylates of Asp74 and Asp90) would have to bind two Mg^{2+} ions with four positive charges. Second, a catalytic metal ion not only has to be *bound* but must also be correctly *positioned*. It appears very unlikely that a second metal ion would bind to the E45A mutant at exactly the same position as in wild type *Eco*RV. Third, the D74A and D90A mutants are catalytically inactive and, hence, differently from E45A, behave exactly as expected for mutants in which a catalytically relevant amino acid residue has been replaced by alanine. Taken together, binding of a catalytical divalent metal ion at the former E45/D74 site in the E45A variant is very unlikely.

Is There Biochemical Evidence for More Than One Catalytically Relevant Divalent Metal Ion Binding Site in the EcoRV−DNA Complex?

The dependencies of DNA cleavage rates by wild type *Eco*RV on the concentrations of Mg^{2+} or Mn^{2+} reported here show a clearly sigmoidal shape that can only be rationalized if at least two functional metal ion binding sites exist, both of which are stimulatory to DNA cleavage. This finding is in perfect agreement with published results obtained by three other experimental approaches, namely, analysis of cleavage rates in the presence of Mn^{2+} and Ca^{2+} (Vipond *et al*., 1995), analysis of metal ion dependence of DNA-binding (Jeltsch *et al*., 1995), and analysis of cleavage of phosphorothioates in the presence of Mg^{2+} or Mn^{2+} (Jeltsch *et al*., 1995). In these studies, however, either special cleavage conditions were employed (presence of Mn^{2+} instead of Mg^{2+}) or only DNA binding was analyzed. Here we have shown that under *normal* cleavage conditions, i.e., in the presence of Mg^{2+}, two metal ions are involved in DNA binding and cleavage by *Eco*RV. The experiments with a heterodimeric *Eco*RV variant in which only one catalytic center is active demonstrate that two Mg^{2+} ions bind to each subunit of *Eco*RV when complexed with DNA.

On the basis of the data presented here, we correct our earlier statement that the DNA cleavage rate of *Eco*RV has a hyperbolic dependence on the Mg^{2+} concentration (Jeltsch *et al*., 1993). As noticed by Vipond *et al*. (1995), this conclusion was drawn on the basis of a model that implies cooperative binding of the metal ions to the *Eco*RV−DNA complex. Here, we have shown that this model indeed does not sufficiently describe the Mg^{2+} dependence of the DNA cleavage rate of *Eco*RV. On the basis of the older data it would have been impossible to distinguish between binding of only one metal ion and non-cooperative binding of two metal ions, because rates were not determined at sufficiently low metal ion concentrations to make this decision.

In addition to the demonstration of two metal ion binding sites that are stimulatory to DNA cleavage, the analysis of the dependence of the DNA cleavage rate on the concentration of Mn^{2+} has provided clear evidence for the existence of at least two additional Mn^{2+} binding sites that are inhibitory to DNA cleavage. As Mn^{2+} is known to bind to DNA in the millimolar concentration range (van Steenwinkel *et al*., 1981; Reid & Cowan, 1990; Black & Cowan, 1994)

and as it is expected that at least some metal ions bound to the DNA substrate have to be displaced by the enzyme, it is likely that these inhibitory metal binding sites are Mn^{2+} binding sites on the DNA substrate.

Is There Biochemical Evidence for a Two-Metal-Ion Mechanism?

A two-metal-ion mechanism would require two metal ions bound at the catalytic center of *Eco*RV. There are two candidates for divalent metal ion binding: the D74/D90 site and the E45/D74 site. Hence, we now have to consider, if the metal ion dependence data provide any hint for an involvement of a metal ion bound by Glu45 and Asp74 in catalysis, in addition to the metal ion bound by Asp74 and Asp90. This analysis, however, has to take into account that other divalent metal ion binding sites exist in the *Eco*RV−DNA complex, one influenced by Tyr219, which stimulates DNA binding, and presumably several others associated with the DNA that are likely to be inhibitory. As shown above, our data clearly indicate that both with Mg^{2+} and Mn^{2+}, *Eco*RV requires at least two metal ions for full catalytic activity. The metal ion dependencies of the two mutants E45A and Y219C should help to find out where these metal ions bind to the *Eco*RV−DNA complex.

In the presence of Mn^{2+} the E45A variant behaves in a qualitatively very similar manner as does wild type *Eco*RV; in particular, this mutant still requires two Mn^{2+} ions for catalytic activity. This result is in full agreement with the good catalytic activity of this mutant. Quantitatively, at least one of the stimulatory and all of the inhibitory metal ion binding sites of E45A have lower affinities than their counterparts in wild type *Eco*RV. The finding that one of the stimulatory Mn^{2+} binding sites in the E45A mutant is weaker than in wild type *Eco*RV might be explained simply by the removal of negative charges from the complex. This stimulatory metal ion which presumably is bound by Asp74 and Asp90 is located only 6 Å away from Glu45, giving rise to a strong electrostatic attraction between this metal ion and Glu45. Given the range of errors of our experiments and data analyses, it is not clear whether the second stimulatory Mn^{2+} binding site has a reduced affinity toward Mn^{2+}. A slightly reduced affinity could be explained by long-range electrostatic effects, because the E45A−DNA complex contains fewer negative charges than a wild type *Eco*RV−DNA complex. For similar electrostatic reasons, one would expect that the DNA is bound more tightly by the E45A mutant that has fewer negative charges in its DNA binding site than wild type *Eco*RV, similarly as observed for the D90A mutant (Thielking *et al*., 1992). This would facilitate the removal of bound counterions, thereby also reducing their apparent inhibition constant, as observed in the experiment.

In contrast to E45A and wild type *Eco*RV, the Y219C variant shows a hyperbolic dependence of its DNA cleavage rate on the concentration of Mg^{2+}. This finding does not prove that this variant needs only one divalent metal ion for catalysis, because additional high-affinity sites could exist that might escape the detection limits of our experiments. The results obtained with the Y219C mutant, however, directly show that the second stimulatory divalent metal ion binding site in *Eco*RV has been lost. Thus, this site is influenced by Tyr219, an amino acid residue located far away from the active center of *Eco*RV. We have shown previously that occupation of this site stimulates specific DNA binding (Jeltsch *et al*., 1995). However, as shown here, it is not directly involved in catalysis. This result strongly suggests that the sigmoidal shape of the curve observed with wild type *Eco*RV is caused by the binding of only one divalent metal ion to the active site of *Eco*RV and binding of one additional metal ion to a site influenced by the Tyr219→Cys substitution. So far we do not know the location of this site with certainty. A long-range effect of Tyr219 on the E45/D74 site is very unlikely in the light of our finding that the E45A mutation has no measurable effect on this site. It is quite likely that the second metal ion binding site is in the vicinity of Tyr219, because with a different experimental approach evidence was produced for a metal ion binding site at the *Eco*RV−DNA interface very close to Tyr219 (at phosphate GpATATC) (Jeltsch *et al*., 1995).

The Y219C mutant cleaves the oligonucleotide substrate under optimum conditions with a rate reduced by only 1 order of magnitude as compared to wild type *Eco*RV. This result means that Mg^{2+} binding to the site influenced by the Y219C mutation is not essential for catalysis. It does, however, stimulate the DNA cleavage rate by about a factor of 10. This finding is interesting as it reminds one that not necessarily an all-or-none effect has to be expected, if functions other than a direct role in catalysis are considered. A similar observation was made for the inhibitory Mn^{2+} binding sites, occupied at higher concentrations of Mn^{2+}. Whereas Mn^{2+} binding at these sites clearly reduces the DNA cleavage rate, it does not prevent DNA cleavage (figure 6a). Thus, again, a gradual rather than an all-or-none effect is observed.

Our results demonstrate that the methodology employed is suited to detect the involvement of more than one divalent metal ion in the catalytic reaction of *Eco*RV and to define likely positions of the binding sites. It has to be mentioned that the Mg^{2+} dependencies of the DNA cleavage rate measured here cannot definitely rule out the binding of two Mg^{2+} ions to the catalytic center of *Eco*RV, because if one binding site had a sufficiently high affinity, it would be occupied under all conditions tested. However, when interpreted in conjunction with our findings that the E45A mutant is catalaytically active and that the stoichiometry of its metal ion interaction is wild type-like, binding of a catalytically important metal ion at the E45/D74 site appears unlikely. It should be pointed out that all biochemical results reported in the literature (Vipond *et al*., 1995; Jeltsch *et al*., 1995), although clearly demonstrating the interaction of the *Eco*RV−DNA complex with more than one divalent metal ion, provide neither any evidence that more than one divalent metal ion is directly involved in the catalytic reaction of *Eco*RV nor that a metal ion bound by Glu45 contributes to catalysis.

Might Other Restriction Endonucleases Employ a Two-Metal-Ion Mechanism for DNA Cleavage?

Four co-crystal structures of type II restriction endonucleases with their DNA substrate have been solved so far [for review see Aggarwal (1995)]: *Eco*RI (Kim *et al*., 1990), *Eco*RV (Winkler *et al*., 1993), *Pvu*II (Cheng *et al*., 1994), and *Bam*HI (Newman *et al*., 1995). These four enzymes have a very similar active site, characterized by a degenerate PD...D/EXK motif (Thielking *et al*., 1991) (in *Bam*HI the lysine residue is replaced by glutamic acid; in *Pvu*II and

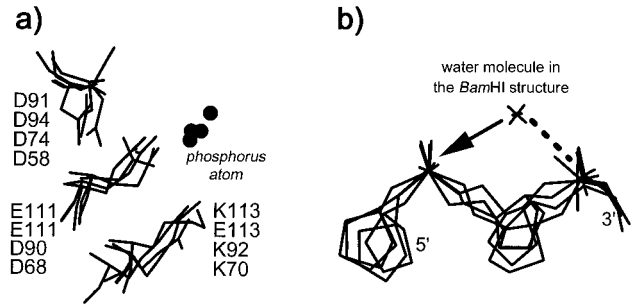

FIGURE 8: Superposition of the catalytically relevant amino acid residues in the active centers of *Eco*RI, *Bam*HI, *Eco*RV, and *Pvu*II. There is no counterpart for *Eco*RV−Glu45 in any of the other enzymes. In a the Cα positions of the catalytic amino acid residues and the positions of the attacked phosphorus atoms are superpositioned. In b the attacked and 3′-adjacent phosphorus atoms are overlaid and in addition one water molecule observed in the *Bam*HI−DNA structure is displayed. Note the similar geometry of all catalytic centers, which suggests a similar catalytic mechanism.

*Bam*HI the proline is absent). As shown in Figure 8a amino acid residues in *Eco*RI, *Bam*HI, and *Pvu*II equivalent to Asp74, Asp90, and Lys92 in *Eco*RV can be accurately superimposed and are positioned very similar as in *Eco*RV with respect to the phosphorus atom attacked during DNA cleavage. Moreover, this phosphorus atom and the 3′ adjacent phosphate group with its *pro*-R_p oxygen atom which plays a decisive role in the substrate assistance model can also be perfectly superimposed (Figure 8b) in the four structures in spite of the fact that the DNA is bent and/or distorted in some of them. This structural similarity is very suggestive for a common mechanism which we propose to be the substrate-assistance mechanism and not the two-metal-ion mechanism. As a matter of fact, in neither *Eco*RI nor *Pvu*II is there an additional acidic amino acid residue that could be considered to be a ligand of a second divalent metal ion. Candidate residues in *Eco*RI (Asp59) and in *Pvu*II (Glu55) were shown by mutational analyses to be dispensable for catalysis (Grabowski *et al*., 1996; H. G. Nastri, I. H. Walker, P. D. Evans, and P. D. Riggs, personal communications). Hence a two-metal-ion mechanism is very unlikely for *Eco*RI and *Pvu*II. Interestingly, in the *Bam*HI structure a water molecule is observed at the hypothetical position of the attacking nucleophile (Newman et al., 1995). This water molecule is hydrogen bonded to the phophate group 3′ to the scissile bond as proposed in the substrate-assistance model for the attacking water.

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