Divalent Metal Ions at the Active Sites of the *Eco*RV and *Eco*RI Restriction Endonucleases†

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ABSTRACT: Restriction enzymes cannot cleave DNA without a metal ion cofactor. The specificities of the *Eco*RV and *Eco*RI endonucleases for metals were studied by measuring DNA cleavage rates with several metal ions and with combinations of metal ions. Both *Eco*RV and *Eco*RI had optimal activities with Mg^{2+}, were less active with several other ions including Mn^{2+}, and had virtually no activity with Ca^{2+}. But the activities of *Eco*RV and *Eco*RI with either Mg^{2+} or Mn^{2+} were perturbed by Ca^{2+}. For *Eco*RI, both Mg^{2+}- and Mn^{2+}-dependent activities, at both cognate and noncognate sites, were all inhibited by Ca^{2+}. The activity of *Eco*RV at its recognition site with Mg^{2+} was also inhibited by Ca^{2+}. But the Mn^{2+}-dependent reaction at the *Eco*RV recognition site was stimulated by Ca^{2+}. *Eco*RV activities at noncognate sites with either Mg^{2+} or Mn^{2+} displayed a biphasic response to Ca^{2+}: stimulation at low concentrations of Ca^{2+} and inhibition at high concentrations. These observations, together with the known structures of the proteins, indicate that *Eco*RI needs only one metal ion per active site and is inactive when Mg^{2+} is displaced by Ca^{2+}, while *Eco*RV needs two and that the displacement of one by Ca^{2+} can enhance activity. We propose a mechanism for phosphodiester hydrolysis by *Eco*RV that involves two metal ions.

Type II restriction endonucleases require divalent metal ions to cleave DNA (Wilson & Murray, 1991). In the absence of divalent metal ions, restriction enzymes can bind to DNA but they cannot cleave it (Roberts & Halford, 1993). The metal is thus likely to be involved in the catalytic reaction, and, for both *Eco*RI and *Eco*RV, metal ions have been located at the active site by X-ray crystallography (Rosenberg, 1991; Kostrewa & Winkler, 1995). But the role of the metal in catalysis has yet to be determined for any restriction enzyme. This report describes a series of kinetic experiments aimed at elucidating the role(s) of metal ions in the *Eco*RV and *Eco*RI endonucleases. The kinetics are correlated to the crystal structures of the enzymes (Kim et al., 1990; Winkler et al., 1993), particularly the structures of their enzyme−substrate or enzyme−product complexes in the presence of divalent metal ions (Rosenberg, 1991; Kostrewa & Winkler, 1995).

The optimal rate for the proper reaction of a type II restriction enzyme, DNA cleavage specifically localized to its recognition site, is obtained with Mg^{2+} as the cofactor. Most restriction enzymes retain some activity with certain alternatives to Mg^{2+}, such as Mn^{2+} or Co^{2+}, though they usually have no activity with other alternatives such as Ca^{2+} or Ba^{2+} (Bennett & Halford, 1989). However, alternative metal ions sometimes perturb both the activity of the restriction enzyme and its specificity (Hsu & Berg, 1978). Restriction enzymes cleave DNA not only at their cognate recognition sites but also, albeit very slowly, at a number of noncognate sites: the latter differ from the recognition sequence by 1 bp[1] (Roberts & Halford, 1993). The substitution of Mg^{2+} by Mn^{2+} can weaken the ability of a restriction enzyme to discriminate cognate from noncognate sites, by reducing reactions rates at cognate sites while increasing those at noncognate sites. For example, with the *Eco*RV endonuclease, the ratios of k_{cat}/K_m values for cognate and noncognate sites are 1×10^6 in the presence of Mg^{2+} and 6 in the presence of Mn^{2+} (Vermote & Halford, 1992).

The two restriction enzymes studied here, *Eco*RI and *Eco*RV, are both dimeric proteins with similar molecular weights and they both cleave DNA specifically at 6 bp palindromic sequences: G↓AATTC for *Eco*RI and GAT↓ATC for *Eco*RV, where ↓ marks the point of cleavage (Hedgpeth et al., 1972; Schildkraut et al., 1984). But in most other respects, *Eco*RV differs radically from *Eco*RI: their amino acid sequences have no homology (Newman et al., 1981; Bougueleret et al., 1984); their structures, as determined by X-ray crystallography on the free proteins and the protein−DNA complexes, show no similarity in either peptide fold or DNA−protein contacts (Kim et al., 1990; Winkler et al., 1993); their mechanisms for DNA recognition differ from each other (Heitman, 1992; Vipond & Halford, 1993).

In the absence of divalent cations, *Eco*RI binds preferentially to its recognition site (Terry et al., 1987) while *Eco*RV binds all DNA sequences with equal affinity (Taylor et al., 1991). For *Eco*RI, the preferential binding makes a large contribution to the specificity of DNA cleavage though it cannot by itself fully account for the specificity (Lesser et al., 1990). In contrast, the specificity of *Eco*RV seems to arise solely in the catalytic reaction (Newman et al., 1990), with a major contribution coming from variations in the affinities of *Eco*RV−DNA complexes for Mg^{2+}: high

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[1] Abbreviations: bp, base pair(s); BME, β-mercaptoethanol; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDTA, (ethylenedinitrolo)tetraacetic acid; K_D, equilibrium dissociation constant.

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affinity for the complex at the recognition site, leading to saturation with Mg^{2+} and thus the maximal rate of DNA cleavage; low affinity for noncognate complexes, resulting in low fractional saturation and thus low cleavage rates (Taylor & Halford, 1989; Vermote & Halford, 1992). The different affinities for Mg^{2+} can be accounted for on the crystal structures of *Eco*RV bound to either specific or nonspecific DNA, the former DNA being highly distorted while the latter has a B-like structure (Winkler et al., 1993). The distortion of the specific DNA inserts the phosphate at the scissile bond into the active site where it forms part of a potential binding site for Mg^{2+} between the protein and the DNA, while the lack of distortion in the nonspecific DNA leaves it distant from the active site.

Despite these differences, the two endonucleases have similar active sites (Winkler, 1992; Selent et al., 1992), and it has been suggested that they employ the same chemical mechanism for phosphodiester hydrolysis (Jeltsch et al., 1993). Both nucleases cleave their targets to leave 5′-phosphates with inverted stereochemistries (Connolly et al., 1984; Grasby & Connolly, 1992). Both active sites contain similar amino acids:

*Eco*RI: Pro90-Asp91...Glu111-Ala112-Lys113

*Eco*RV: Pro73-Asp74...Asp90-Ile91-Lys92

Apart from the proline, which is *trans* in *Eco*RI and *cis* in *Eco*RV (Winkler et al., 1993), these amino acids are arranged in similar fashions around the DNA phosphate at the scissile bond. Furthermore, the active sites of *Bam*HI and *Pvu*II contains aspartate and glutamate residues in analogous positions to Asp91 and Glu111 in *Eco*RI though *Bam*HI differs from *Eco*RI by having a glutamate in place of Lys113 (Newman et al., 1994; Cheng et al., 1994). Mutational analyses in *Eco*RI, *Eco*RV, and *Bam*HI have shown that these residues are essential for catalytic activity (King et al., 1989; Thielking et al., 1991; Selent et al., 1992; Dorner & Schildkraut, 1994). The carboxylate functions of Asp91 and Glu111 in *Eco*RI provide ligands for binding Mg^{2+} (Rosenberg, 1991). Similarly, both Asp74 and Asp90 coordinate the metal in the enzyme−substrate complex for *Eco*RV (Kostrewa & Winkler, 1995). However, *Eco*RV contains an additional carboxylate at its active site, Glu45, that has no counterpart in *Eco*RI. The active site in *Eco*RV thus has two potential binding sites for divalent metal ions: one between Asp90 and Asp74 and another between Asp74 and Glu45 (Winkler et al., 1993). The latter binds Mg^{2+} in the enzyme−product complex for *Eco*RV (Kostrewa & Winkler, 1995).

EXPERIMENTAL PROCEDURES

Proteins. *Eco*RI and *Eco*RV endonucleases were purified as described previously (Luke et al., 1984, 1987). The concentrations of both enzymes are given in terms of the dimeric proteins.

DNA. The following plasmids were used: pAT153 (Twigg & Sherratt, 1980), pAT153b (Taylor & Halford, 1989), and pAT153c (this study). The latter differs from pAT153 by a 185 bp deletion between its *Eco*RI and *Eco*RV sites that removes both of these sites. It was constructed by cleaving pAT153 with *Eco*RI and *Eco*RV, filling in the *Eco*RI ends with Klenow polymerase, and then recircularizing the large fragment by blunt-end ligation. The plasmids were used to transform *Escherichia coli* HB101. The transformants were grown in M9 minimal media containing 1 mCi/L [*methyl*-^{3}H]thymidine. The supercoiled form of each plasmid was purified on CsCl/ethidium bromide gradients as before (Halford & Goodall, 1988).

Reactions. Kinetics of DNA cleavage were measured by adding the appropriate amount of restriction enzyme to 10 nM ^{3}H-labeled plasmid (>80% supercoiled DNA) at 25 °C in one of the buffers listed below. Typically, 10 samples were taken from the mixture at timed intervals, and the reaction in each sample was stopped immediately with EDTA. The samples were then analyzed by electrophoresis through agarose to separate the supercoiled, open circle, and linear forms of the plasmid, and the concentrations of all three forms were measured by scintillation counting (Halford & Goodall, 1988). For reactions at recognition sites, the buffers were *Eco*RV buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM BME, 100 μg/mL BSA, pH 7.5) or *Eco*RI buffer (100 mM Tris-HCl, 50 mM NaCl, 6 mM BME, 100 μg/mL BSA, pH 7.5), supplemented with divalent metal ion chlorides at the concentrations noted for each reaction. Solutions of each metal ion chloride in water were from regular analytical grade solids (BDH, Poole, U.K.) and were used without further purification: they are likely to contain other divalent metal ions at ≤0.01% of the concentration of the desired metal. The same buffers were also used for reactions at noncognate sites with $MnCl_2$. Reactions at noncognate sites with $MgCl_2$ were done in buffer C: 50 mM Tris-HCl, 100 mM NaCl, 10 mM BME, 100 μg/mL BSA, and 10% (v/v) DMSO, pH 8.5 (Taylor & Halford, 1989).

Whenever possible, the reactions were carried out under steady-state conditions with the enzyme concentration at least 5 times lower than the concentration of the plasmid. Zero-order reaction velocities were evaluated from the period in the reaction during which concentration of the supercoiled DNA substrate decreased linearly with time. [The observed velocities correspond to V_{max} values since the substrate concentrations were much higher than the K_m values of either *Eco*RI or *Eco*RV for their respective recognition sites on pAT153 (Terry et al., 1987; Halford & Goodall, 1988).] Reaction velocities were normalized against the enzyme concentration to give k_{cat} values. Some of the reactions reported here were too slow to measure under steady-state conditions, and these were studied with the enzyme in excess of the DNA. In these reactions, the decline in the concentration of the supercoiled DNA substrate followed an exponential progress curve from which a first-order rate constant (k_a) was calculated. The values of k_a were normalized against the enzyme concentration to give a parameter, $k_a/[E_0]$, that is related to k_{cat}/K_m (Taylor & Halford, 1989; Vermote & Halford, 1992). All of the values for k_{cat} in Figures 1 and 2, and for $k_a/[E_0]$ in Figure 3, are averages from two or more repeat experiments.

RESULTS

Metal Ion Specificity. The recognition sequences for *Eco*RV and *Eco*RI each appear once in the DNA sequence of pAT153. The monomeric form of pAT153 (3685 bp) was isolated from a *rec*A strain of *E. coli*, as a covalently closed circle of supercoiled DNA, and this was used as a substrate for the *Eco*RV and *Eco*RI endonucleases in the presence of several divalent metal ion chlorides, each at 1 mM concen-

trations. The reactions were monitored by using agarose gel electrophoresis to separate the DNA substrate from the cleaved products. Both enzymes cut their respective recognition sites in both strands of the duplex, to convert the supercoiled substrate to the full-length linear form of the DNA, but open circle DNA, produced by cutting one strand of the duplex, was seen as an intermediate in the reactions with some metal ions (data not shown). The reactions in the presence of each cation were studied with a range of enzyme concentrations (from 1 nM to 1 μM) and over varied time periods (10 min to 6 h) so that the relative activities with each metal ion could be estimated.

With the *Eco*RV endonuclease, the relative activities fell according to the series

$$Mg^{2+} > Co^{2+} > Mn^{2+} \gg Cd^{2+} > Zn^{2+} > Ni^{2+} \gg Ca^{2+}$$

where > indicates a decrease by less than 10-fold from the preceding cation and ≫ a decrease by more than 10-fold. This series confirms and extends a previous study (Luke et al., 1987). A similar series was obtained with the *Eco*RI endonuclease:

$$Mg^{2+} > Mn^{2+} \approx Co^{2+} \gg Zn^{2+} \gg Cd^{2+} > Ni^{2+} \gg Ca^{2+}$$

However, the decreases in activity caused by replacing Mg^{2+} with another metal ion were generally larger for *Eco*RI than *Eco*RV. Mn^{2+} and Zn^{2+} were exceptions: they both gave more activity with *Eco*RI than with *Eco*RV. In the presence of Ca^{2+}, some DNA cleavage was observed in prolonged reactions with high concentrations of the *Eco*RV enzyme but none at all with *Eco*RI. We estimate that the activity of *Eco*RV is at least 1×10^5 times lower in Ca^{2+} than Mg^{2+} and that the upper limit for the activity of *Eco*RI in Ca^{2+} is 1×10^6 times lower than that with Mg^{2+}. However, we cannot eliminate the possibility that the low levels of activity seen with Ca^{2+} are due to contaminating metal ions in the solutions of $CaCl_2$.

Ca^{2+} and Mg^{2+} or Mn^{2+} in Cognate Reactions. In order to determine whether the *Eco*RI and *Eco*RV endonucleases can interact with Ca^{2+}, despite its failure to support DNA cleavage, the rates at which *Eco*RI and *Eco*RV cleaved their recognition sites on pAT153 were measured in buffers containing both $MgCl_2$ and $CaCl_2$ (Figure 1). The reactions were carried out under steady-state conditions. In the presence of both Mg^{2+} and Ca^{2+}, as in previous studies with Mg^{2+} alone (Terry et al., 1987; Halford & Goodall, 1988), both endonucleases made double-strand breaks at their recognition sites to convert the supercoiled form of pAT153 directly to the linear form, without the accumulation of open circle intermediates (data not shown). However, in reactions with a fixed concentration of $MgCl_2$ and varied concentrations of $CaCl_2$, the rates at which both *Eco*RI and *Eco*RV cleaved their recognition sites decreased steeply as the concentration of $CaCl_2$ was increased (Figure 1a,b).

Similar experiments were also carried out to measure the effect of Ca^{2+} on the Mn^{2+}-dependent reactions of the *Eco*RI and *Eco*RV endonucleases at their respective recognition sites on pAT153 (Figure 2). The *Eco*RI reactions with Mn^{2+} as the cofactor were inhibited by increasing levels of Ca^{2+} (Figure 2a), just as they had been when Mg^{2+} was the cofactor (Figure 1a). But the effect of Ca^{2+} on *Eco*RV and Mn^{2+} was the opposite to that observed with *Eco*RV and

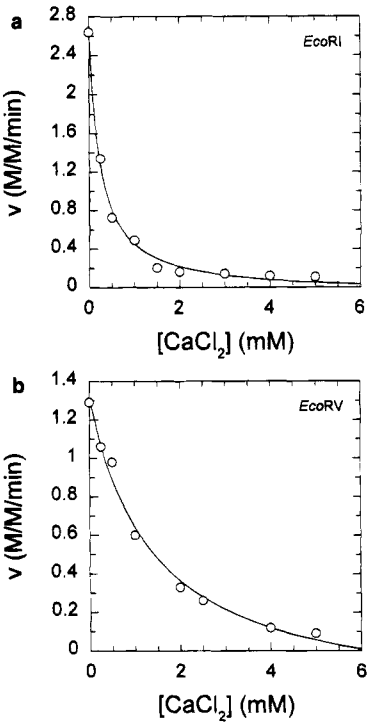


FIGURE 1: Cleavage of recognition sites by (a) *Eco*RI and (b) *Eco*RV with Mg^{2+} and Ca^{2+}. Values of k_{cat} were determined from the steady-state reactions of either the *Eco*RI endonuclease (0.1−2 nM) or the *Eco*RV endonucleases (0.5 nM) on 10 nM pAT153 in the presence of Mg^{2+} ions. The reactions were in either *Eco*RI buffer or *Eco*RV buffer, supplemented in both cases with 5 mM $MgCl_2$ and the amount of $CaCl_2$ as shown. (Panel a) *Eco*RI. (Panel b) *Eco*RV.

Mg^{2+}. Instead of inhibiting the reaction (Figure 1b), the addition of Ca^{2+} to the *Eco*RV restriction enzyme in the presence of Mn^{2+} led to enhanced rates of DNA cleavage (Figure 2b). The increase was linearly proportional to the concentration of Ca^{2+} ions, but it also depended on the ratio of $[Ca^{2+}]$ to $[Mn^{2+}]$: with double the amount of Mn^{2+}, the degree of stimulation by Ca^{2+} was halved (Figure 2b). Moreover, this effect on the Mn^{2+}-dependent activity of *Eco*RV was found to be specific to Ca^{2+} ions. The seven divalent metal ions, used above to test individually for *Eco*RV activity, were also tested in all possible pairwise combinations. No pair, other than Mn^{2+} and Ca^{2+}, had this synergistic effect on *Eco*RV where the activity with two metal ions was higher than with either metal alone (data not shown). None of the pairs had a synergistic effect on *Eco*RI.

Ca^{2+} and Mg^{2+} or Mn^{2+} in Noncognate Reactions. The *Eco*RV enzyme cleaves pAT153 not only at its recognition site but also at 12 noncognate sites, each of which differs from the cognate site by 1 bp (Halford et al., 1986). But one noncognate site on pAT153, GTTATC at position 1734, is cleaved by *Eco*RV faster than any other (Taylor & Halford, 1992). The plasmid pAT153b is identical to pAT153 apart from an 8 bp insertion that disrupts the *Eco*RV recognition site, so the initial reaction of *Eco*RV on pAT153b is at the preferred noncognate site. Under standard buffer conditions with 1 nM enzyme, the reaction at the noncognate site is too slow to detect, but it can readily be detected either by using enzyme concentrations > 1 μM or by performing it in the presence of DMSO or $MnCl_2$ (Taylor & Halford, 1989; Vermote & Halford, 1992). *Eco*RV cleaves its preferred noncognate site on pAT153b first in one strand, converting

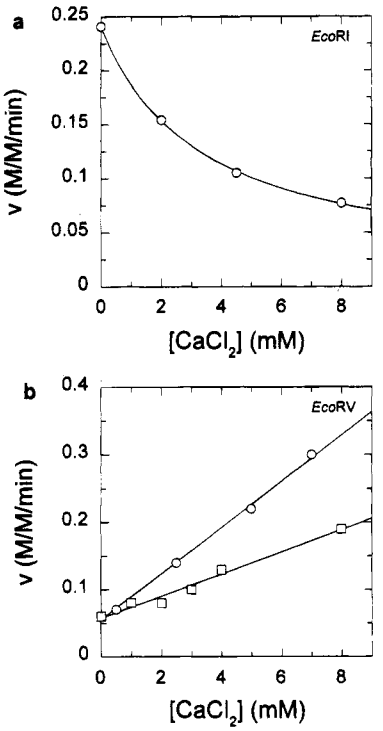


FIGURE 2: Cleavage of recognition sites by (a) *Eco*RI and (b) *Eco*RV with Mn^{2+} and Ca^{2+}. Values of k_{cat} were determined from steady-state reactions of either the *Eco*RI endonuclease (0.2−1 nM) or the *Eco*RV endonucleases (1 nM) on 10 nM pAT153 in the presence of Mn^{2+} ions. The *Eco*RI reactions (panel a) were in *Eco*RI buffer, supplemented with 0.5 mM $MnCl_2$ and the amount of $CaCl_2$ shown. The *Eco*RV reactions (panel b) were in *Eco*RV buffer, supplemented with either 0.5 mM $MnCl_2$ (circles) or 1 mM $MnCl_2$ (squares) and the amount of $CaCl_2$ shown.

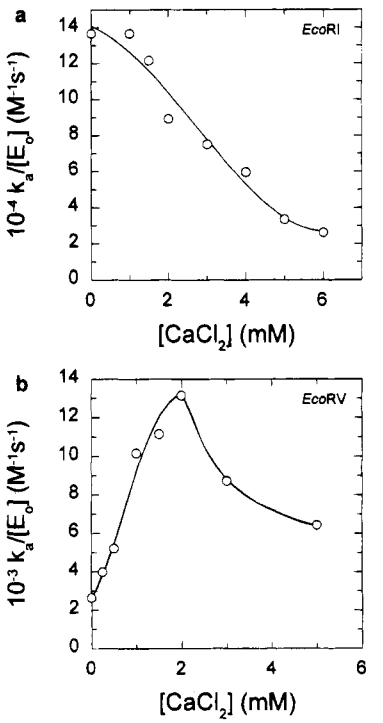


FIGURE 3: Cleavage of noncognate sites by (a) *Eco*RI and (b) *Eco*RV with Mg^{2+} and Ca^{2+}. Values of $k_a/[E_0]$ were determined from the decrease in the concentration of supercoiled DNA during the reactions of either the *Eco*RI endonuclease (20 nM) on 10 nM pAT153c (panel a) or the *Eco*RV endonuclease (100 nM) on 10 nM pAT153b (panel b). The reactions with both *Eco*RI and *Eco*RV were carried out in buffer C supplemented with 5 mM $MgCl_2$ and the amount of $CaCl_2$ shown.

the supercoiled DNA to the open circle form, and then in the second strand to produce linear DNA. In this study, the reactions of *Eco*RV at noncognate sites were carried out with enzyme in excess of the DNA, and the rates ($k_a/[E_0]$ values) were evaluated from the exponential decline in the concentration of supercoiled DNA. Similarly, pAT153 contains several sites that differ from the *Eco*RI recognition site by 1 bp. *Eco*RI reactions at noncognate sites were measured under the same conditions as above with pAT153c as the substrate, a derivative that lacks the cognate site for *Eco*RI (Experimental Procedures). However, the rate measured for *Eco*RI reactions at noncognate sites is a composite rate since the conversion of supercoiled pAT153c to open circles can be due to a single-strand break at any one of the noncognate sites.

The effects of Ca^{2+} on the reactions at noncognate sites were studied by measuring the rate at which *Eco*RI nicked pAT153c in the presence of both $MgCl_2$ and $CaCl_2$ and likewise for the rate at which *Eco*RV nicked pAT153b (Figure 3). The *Eco*RI reaction was inhibited by concentrations of $CaCl_2$ above 1 mM, in a fashion similar to that observed in the cognate reaction of *Eco*RI (Figure 1a), though concentrations of $CaCl_2$ below 1 mM had no significant effect on the rate of nicking at noncognate sites in the presence of 5 mM $MgCl_2$ (Figure 3a).[2] In contrast, the rate of the Mg^{2+}-dependent reaction of *Eco*RV at its noncognate site on pAT153b initially increased with increasing concentrations of $CaCl_2$, but further increases in the level of Ca^{2+} then caused a progressive reduction in rate (Figure 3b). Further experiments were carried out to analyze the effect of Ca^{2+} on the reactions of *Eco*RI and *Eco*RV at noncognate sites, but with Mn^{2+} as the cofactor for DNA cleavage instead of Mg^{2+}. For both *Eco*RI and *Eco*RV, the results with Mn^{2+} were similar to those in Figure 3 with Mg^{2+}. Ca^{2+} inhibited the Mn^{2+}-dependent reactions of *Eco*RI at noncognate sites but again gave a dual response in the *Eco*RV reactions, with low concentrations of $CaCl_2$ enhancing the rate (up to a maximum at 2 mM $CaCl_2$) and higher concentrations reducing the rate (data not shown).

The *Eco*RV restriction enzyme thus shows three different responses to Ca^{2+} ions, depending, first, on whether the reaction is at the *Eco*RV recognition site or at a noncognate site and, second, on whether the cofactor for DNA cleavage is Mg^{2+} or Mn^{2+}. The only *Eco*RV reactions that are affected similarly by Ca^{2+} are the Mg^{2+}- and the Mn^{2+}-dependent cleavages at noncognate sites. However, Ca^{2+} has essentially the same effect on all of *Eco*RI reactions studied here, inhibiting those at both cognate and noncognate sites with either Mg^{2+} or Mn^{2+} as the cofactor.

[2] While concentrations of Ca^{2+} ions > 1 mM had no significant effect on the rate at which *Eco*RI converted supercoiled pAT153c to open circles (Figure 3a), low levels of Ca^{2+} (≤0.5 mM) accelerated the conversion of the open circle form to linear DNA. To linearize pAT153c, the *Eco*RI endonuclease must cut both strands of the duplex at one noncognate site, so the effect of Ca^{2+} on enhancing second strand cleavage, without affecting first strand cleavage, is probably due to increased processivity. This increase might be caused by an intermediate with Ca^{2+} bound to one subunit of the dimeric enzyme and Mg^{2+} to the other: an interaction between Ca^{2+} and the phosphomonoester in the nicked strand could perhaps lock the enzyme onto the DNA at that site, thus enabling the subunit with Mg^{2+} to cut the second strand.

DISCUSSION

Both the *Eco*RV and the *Eco*RI endonucleases cleave their recognition sequences optimally in the presence of Mg^{2+} though neither enzyme is completely specific for Mg^{2+}. A number of other divalent metal ions, including Co^{2+} and Mn^{2+}, support lower rates of DNA cleavage. But despite the chemical similarity of Mg^{2+} and Ca^{2+} (Glusker, 1991), both *Eco*RV and *Eco*RI are virtually inactive with Ca^{2+}. Two simple models can be proposed for why these endonucleases have near-zero activities with Ca^{2+}. One is that Ca^{2+} fails to bind to the active site. It might be excluded from a site for Mg^{2+} by means of its larger ionic diameter (Glusker, 1991). But if Ca^{2+} fails to bind, then the addition of this ion should have no effect on either the Mg^{2+}- or the Mn^{2+}-dependent activities of the restriction enzyme, yet we observed large changes in the activities of both nucleases upon the addition of Ca^{2+} (Figures 1−3).

It is possible that the influence of Ca^{2+} on the activities of *Eco*RI and *Eco*RV were due to secondary effects on protein or DNA conformation or to changes to the general ionic strength of the solutions. The latter can be excluded: all of the reactions described here were carried out with Tris and NaCl concentrations that total 0.15 M so the addition of ≤ 5 mM $CaCl_2$ will make little difference to the ionic strength. In addition, it is unlikely that a secondary effect from DNA structure could cause the observed changes in DNA cleavage activity. Both Ca^{2+} and Mg^{2+} bind to DNA exclusively at the phosphates, in contrast to some of the other metal ions tested here, such as Mn^{2+}, that bind to DNA at both the phosphates and the bases (Bloomfield et al., 1974). The impact of Ca^{2+} on the overall structure of DNA is very similar to that of Mg^{2+} (Bauer, 1978). Moreover, the effects of Ca^{2+} were competitive with either Mg^{2+} or Mn^{2+}: they depended on the ratios of the two sorts of metal ions.

The second model is that Ca^{2+} binds to the active sites of *Eco*RV and *Eco*RI but fails to support the catalytic reaction. This model predicts that Ca^{2+} should inhibit the reactions with either Mg^{2+} or Mn^{2+} by displacing the active cofactor. All of the data on the *Eco*RI endonuclease presented here are consistent with this second model. For *Eco*RI, the simplest mechanism to account for the reductions in DNA cleavage rates is a direct 1:1 competition between Ca^{2+} and either Mg^{2+} or Mn^{2+} for a single binding site at the catalytic center. The ratio of $[Ca^{2+}]$ to $[Mn^{2+}]$ that is needed to reduce the rate of the Mn^{2+}-dependent reaction to half of its maximum is >1 (Figure 2a). This indicates that *Eco*RI has a lower affinity for Ca^{2+} than Mn^{2+}. But the Mg^{2+}-dependent reaction has half of its maximal rate with a ratio of $[Ca^{2+}]$ to $[Mg^{2+}]$ that is <1 (Figure 1a). Thus, far from excluding the larger Ca^{2+} ion from its active site, *Eco*RI appears to have a higher affinity for Ca^{2+} than Mg^{2+}. This proposal for a single binding site for a metal ion at each center is consistent with the crystallography on *Eco*RI. Soaking Mn^{2+} into the co-crystals of *Eco*RI bound to its recognition sequence results in the binding of one Mn^{2+} ion at each active site and concomitant cleavage of the DNA duplex: in the enzyme−product complex, the Mn^{2+} ion is coordinated by the carboxylates of Asp91 and Glu111 and by one of the oxygens from the 5′-phosphate (Rosenberg, 1991; J. M. Rosenberg, personal communication).

In pancreatic DNase I and in staphylococcal nuclease, the metal ion appears to promote phosphodiester hydrolysis by interacting with a nonbridging oxygen on the scissile phosphodiester, thus polarizing the phosphate to facilitate a nucleophilic attack at the phosphorus (Saenger, 1991; Suck, 1992). The same mechanism has also been proposed for *Eco*RI [reviewed by Heitman (1992)]. But this chemical function can be met by either Mg^{2+} or Ca^{2+} (Gerlt, 1993), yet *Eco*RI specifically uses Mg^{2+} while staphylococcal nuclease achieves the same end solely with Ca^{2+}. It is difficult to explain why *Eco*RI bound to Ca^{2+} fails to cleave DNA. The reaction of *Eco*RI seems to require a very precise alignment of the catalytic functions in the protein, the metal ion and the DNA (Jeltsch et al., 1993). Perhaps only Mg^{2+} gives the correct alignment, while Mn^{2+} produces a slightly incorrect alignment and Ca^{2+} a gross misalignment.

In contrast to *Eco*RI, the behavior of the *Eco*RV endonuclease with Ca^{2+} cannot be fitted to either of the two simple models discussed above. Depending on the nature of the reaction, Ca^{2+} can act as an inhibitor of *Eco*RV (Figure 1b) or as an activator (Figure 2b) or both (Figure 3b). One possibility for activation by Ca^{2+} is a binding site for this metal somewhere in the protein outside the active site, as is the case with DNase I (Suck, 1992). But this cannot explain both activation and inhibition. Nor is there any obvious location in the three-dimensional structure of the *Eco*RV protein (Winkler et al., 1993) for an external binding site for Ca^{2+}. However, many nucleases contain two or more metal ions at their active sites. Examples include the 3′ → 5′ exonuclease of DNA polymerase I (Derbyshire et al., 1991; Beese & Steitz, 1991), nuclease P1 (Volbeda et al., 1991), the RNase H domain of HIV reverse transcriptase (Davies et al., 1991), and certain ribozymes (Steitz & Steitz, 1993). *Eco*RV could be another example.

The effects of Ca^{2+} on *Eco*RV can be explained if each catalytic center in the dimeric protein contains two binding sites for divalent metal ions, which we shall call A and B, and that the B site must be filled by an appropriate metal ion (Mg^{2+} or Mn^{2+}) for catalytic activity.[3] In the following qualitative discussion, the metal ions will be specified by notations such as E-Ca^{2+}-Mg^{2+}, where the first-named metal is at the A site and the second at the B site. The discussion is necessarily qualitative because the occupancies of sites A and B by either Mg^{2+}, Mn^{2+}, or Ca^{2+} will be determined by the relative affinities for each ion but these may differ between A and B and they will also vary depending on whether the *Eco*RV protein is bound to cognate or noncognate DNA (Halford et al., 1993). Thus, a complete desription of this system would require at least 12 K_D values: the constants for binding Mg^{2+}, Mn^{2+}, and Ca^{2+} to the A site and to the B site in the presence of either cognate or noncognate DNA.

The activation of cleavage at the *Eco*RV recognition site with Mn^{2+} as the cofactor (Figure 2b) could be due to the replacement of Mn^{2+} by Ca^{2+} at site A but not at B, to give an E-Ca^{2+}-Mn^{2+} complex that is more active than the E-Mn^{2+}-Mn^{2+} complex. The 3′ → 5′ exonuclease of DNA polymerase I also shows enhanced activities with certain combinations of metal ions though the degree of enhancement, 1.5-fold (Derbyshire, 1992), is less than the 5-fold enhancement of *Eco*RV seen here with both Mn^{2+} and Ca^{2+}. In the presence of Mn^{2+}, the rate of the *Eco*RV reaction increased linearly with increasing amounts of $CaCl_2$, which implies that the affinity for Ca^{2+} at site A is much lower than that for Mn^{2+} and that only a small fraction of the A

sites bind Ca^{2+} ions. The maximal enhancement, at a concentration of $CaCl_2$ that is sufficiently high to displace Mn^{2+} from all of the A sites (but none of the B sites), would be even larger than the 5-fold effect shown in Figure 2b.

The *Eco*RV endonuclease has a lower affinity for Mg^{2+} than Mn^{2+}: mixtures of $MgCl_2$ and $MnCl_2$ give the same rates as $MnCl_2$ alone, even when the ratio of $[Mg^{2+}]$ to $[Mn^{2+}]$ is 20:1 (Vermote & Halford, 1992). Consequently, in the Mg^{2+}-dependent reaction at the *Eco*RV recognition site (Figure 1b), calcium ions may inhibit DNA cleavage by displacing Mg^{2+} from both sites A and B.

The same mechanism can also account for both the initial activation of *Eco*RV reactions at noncognate sites by low concentrations of $CaCl_2$ and the inhibition of these reactions at high concentrations of $CaCl_2$ (Figure 3b): by first displacing Mg^{2+} from site A to give an E-Ca^{2+}-Mg^{2+} complex with enhanced activity and then, at higher $CaCl_2$ concentrations, displacing Mg^{2+} from both A and B to give an E-Ca^{2+}-Ca^{2+} complex that has no activity. The effect of Ca^{2+} on the Mn^{2+}-dependent reactions of *Eco*RV at noncognate sites was the same as that for the Mg^{2+}-dependent reactions, but this pattern of initial enhancement followed by inhibition (Figure 3b) differed from the pattern seen with Mn^{2+} at the cognate site where only the enhancement was observed (Figure 2b). However, the affinity of the *Eco*RV endonuclease for Mn^{2+} is lower when the protein is bound to noncognate DNA than when it is bound to cognate DNA (Halford et al., 1993). Consequently, in the noncognate reactions of *Eco*RV, the E-Ca^{2+}-Mn^{2+} complex with enhanced activity may be converted, at high concentrations of $CaCl_2$, to an inactive E-Ca^{2+}-Ca^{2+} complex.

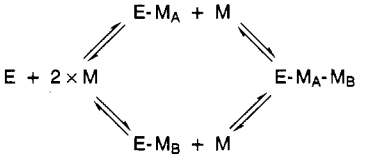
[3] The reaction rate of *Eco*RV on an oligonucleotide substrate has a hyperbolic dependence on the concentration of Mg^{2+} ions, and this was taken to suggest that each active site in *Eco*RV has only one Mg^{2+} ion (Jeltsch et al., 1993). The grounds for this suggestion were that, if the only active form of the enzyme was that with two metals bound per subunit (E-M-M), the relative activity (v/V_{max}) would vary with the concentration of $MgCl_2$ (given as [M]) according to

$$v/V_{max} = [M]^2/([M]^2 + K_D)$$

where K_D, the equilibrium dissociation constant for Mg^{2+}, is given by

$$K_D = \{[E] \times [M]^2\}/[E\text{-}M\text{-}M]$$

The rate would then have a sigmoidal dependence on the concentration of $MgCl_2$ with a Hill coefficient of 2. However, the above equations are only valid if the two metal ions bind with infinite cooperativity. If two metal ions bind independently to separate sites, A and B,



and if only the E-M_A-M_B complex is active, the correct equation for the variation in reaction velocity with the concentration of $MgCl_2$ is

$$v/V_{max} = [M]^2/\{([M] + K_A) \times ([M] + K_B)\}$$

where K_A and K_B are the K_D values for sites A and B. The latter equation approaches a rectangular hyperbola, defined by the weaker site, whenever $K_A > K_B$ or vice-versa. It predicts a sigmoidal relationship only when K_A and K_B are similar to each other, but, even when $K_A = K_B$, the deviations from a hyperbola are slight and never amount to ≥4% of the maximal velocity. Contrary to Jeltsch et al. (1993), the concentration dependence of the cleavage rate cannot reveal the number of metal ions per active site.

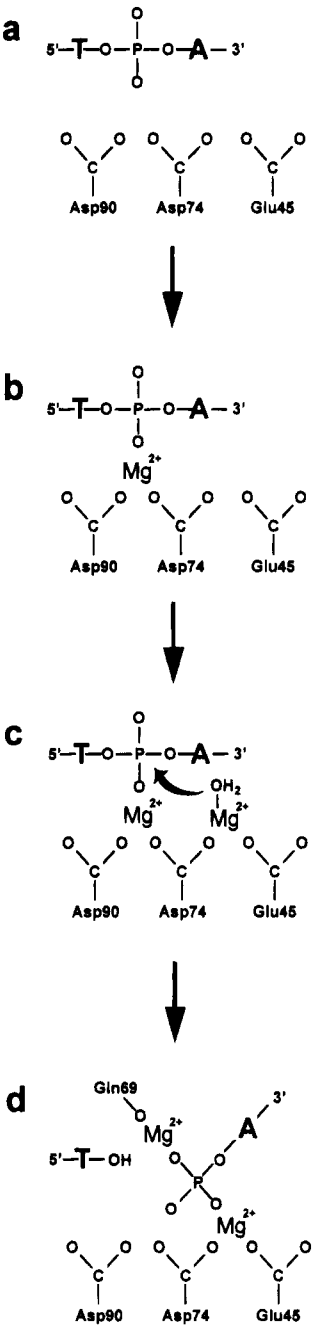


FIGURE 4: Proposal for the chemical mechanism of phosphodiester hydrolysis by *Eco*RV. The first, second, and fourth cartoons (a, b, and d) are representations of the active site in the *Eco*RV endonuclease, as determined by X-ray crystallography (Kostrewa & Winkler, 1995) on: (a) the crystals of *Eco*RV bound to its recognition sequence in the absence of Mg^{2+}, (b) the crystals in panel a after soaking with Mg^{2+}, (d) the crystals obtained after the Mg^{2+}-dependent cleavage reaction. The cartoons show the TpA step in the DNA sequence GATATC that is cleaved by *Eco*RV, the Mg^{2+} ions, and the groups in the protein that ligand the metal ions. We propose an intermediate between b and d, represented by the third cartoon (c), in which the phosphorous at the TpA step is attacked by a molecule of water attached to the Mg^{2+} ion between Asp74 and Glu45.

Where are these metal ions located in the *Eco*RV protein? The active site in *Eco*RV contains three carboxylates, Asp90, Asp74, and Glu45, that form two binding sites for divalent metals (Figure 4a): one between Asp90 and Asp74 (which we shall call the 90/74 site) and another between Asp74 and Glu45 (the 74/45 site). The former is similar to the single

site in *Eco*RI while latter has no equivalent in *Eco*RI (Winkler et al., 1993). Soaking the co-crystals of *Eco*RV bound to its specific DNA with either Mg^{2+} or Ca^{2+} resulted in the incorporation of the metal at only the 90/74 site (Kostrewa & Winkler, 1995): the metal at this site was coordinated not only by the carboxylate functions of Asp90 and Asp74 but also by the phosphodiester at the scissile bond in the DNA (Figure 4b). This location is essentially as had been predicted from the structure of the enzyme−DNA complex in the absence of Mg^{2+} since a metal between Asp74 and Glu45 would be too far away from the scissile bond to allow it to interact directly with the phosphate (Winkler et al., 1993). However, in contrast to *Eco*RI where the addition of Mn^{2+} to the enzyme−DNA co-crystals lead to DNA cleavage (Rosenberg, 1991), no DNA cleavage was observed when Mg^{2+} bound to the 90/74 site in the *Eco*RV−DNA complex (Kostrewa & Winkler, 1995). Though crystallization may have prevented a conformational transition in *Eco*RV required for catalysis, a further possibility is that the binding of Mg^{2+} to the 90/74 site is insufficient for the reaction and an additional metal ion is needed.

In the complex of *Eco*RV bound to its products after DNA cleavage, the 90/74 site is no longer occupied by Mg^{2+}. Instead, a Mg^{2+} ion is located at the 74/45 site and this Mg^{2+} interacts with the 5'-phosphate in the product (Figure 4d). The comparison of the structures for the enzyme−substrate and enzyme−product complexes thus leads to the impression that the metal ion translocates from the 90/74 site to the 74/45 site during the course of the reaction and is followed by the DNA phosphate: the 5′-phosphate in the cleaved DNA is located 4 Å away from its position in the intact DNA (Kostrewa & Winkler, 1995). [The 5′-phosphate in the cleaved DNA also interacts with a second metal coordinated to Gln69. But we suspect that the metal on Gln69 has no catalytic function. The main chain carbonyl group of Gln69 is the only group in the protein that interacts with this metal ion and, since 1 main chain carbonyl group is unlikely by itself to form a specific binding site for Mg^{2+}, this site may exist only after the cleavage reaction has been completed. However, the metal at Gln69 may still influence k_{cat} by affecting the rate of product dissociation.]

In order to account for the differences in the structures of the enzyme−substrate and enzyme−product complexes for *Eco*RV, we propose that the reaction proceeds through a transient intermediate in which both the 90/74 and the 74/45 sites are simultaneously filled by Mg^{2+} (Figure 4c). This proposal can also account for the kinetics of *Eco*RV with combinations of metal ions (Figures 1−3), with site A as the 90/74 location and site B 74/45. In addition, the stopped-flow experiments on *Eco*RV in the following paper show that the binding of Mg^{2+} occurs at two separate stages in the reaction pathway: one at a high affinity site prior to DNA cleavage and another at a low affinity site concomitant with cleavage (Baldwin et al., 1995). The metal may bind first to the 90/74 site: either Mg^{2+}, Mn^{2+}, or Ca^{2+} at this site could polarize the phosphate in readiness for a nucleophilic attack. But the attack seems not to occur until the 74/45 site is also filled with Mg^{2+} or Mn^{2+} (but not Ca^{2+}).

The attacking nucleophile could be a molecule of water coordinated to the Mg^{2+} at the 74/45 site (Figure 4c). This proposal is consistent with the known stereochemistry of the *Eco*RV reaction (Grasby & Connolly, 1992). However, the water would have to be activated by some means. If the activation is by deprotonization to hydroxide, as has been suggested for Klenow exonuclease (Steitz, 1993), the pK_a of *aquo*-Mg^{2+} at the active site would have to be reduced from its value in free solution, 11.4 (Cotton & Wilkinson, 1988). But since the active site of the *Eco*RV endonuclease contains a large number of immobilized water molecules (Kostrewa & Winkler, 1995), the proton-transfer steps will be difficult to define as they will be analogous to proton transfer through ice. The attack by a ligand on the second Mg^{2+} would effectively transfer the phosphate from the 90/74 site to the 74/45 site and the Mg^{2+} at the first site could then dissociate from the protein. However, the insertion of a metal ion at the vacant 74/45 site in the enzyme−substrate complex for *Eco*RV would leave the water bound to that metal ion over 4 Å away from the phosphorous at the scissile bond, too far for a nucleophilic attack. But at the transition state, the phosphorus will presumably be located somewhere between its positions in the enzyme−substrate and the enzyme−product complexes (Kostrewa & Winkler, 1995).

The mechanism proposed here (Figure 4) is also consistent with the effects of mutating the amino acids at positions 45, 74, and 90 in *Eco*RV (Thielking et al., 1991; Selent et al., 1992). Mutant proteins with Ala in place of either Asp74 or Asp90 had no detectable activity while the substitution of Glu45 by Ala caused a 10 000-fold decrease in activity. The conservative change of Asp to Glu at position 90 had no effect on activity while the equivalent change from Glu to Asp at position 45 resulted in a 300-fold decrease in k_{cat} (Selent et al., 1992). On the plasmid that Selent et al. (1992) used as the substrate for the mutant proteins, the rate-limiting step in the reaction of wild-type *Eco*RV is product dissociation (Halford & Goodall, 1988). Thus, a 300-fold decrease in k_{cat} is probably due to a much larger decrease in the rate of phosphodiester hydrolysis. One proposal for the mechanism of *Eco*RV assigns key roles to Asp74 and Asp90 but not to Glu45 (Jeltsch et al., 1993), but all three of these amino acids appear to be essential. The adjacent phosphodiester on the 3′-side of the scissile bond also appears to be essential for *Eco*RV activity (Jeltsch et al., 1993), but this may be analogous to the "phosphate clamps" in *Eco*RI (Lesser et al., 1990).

Regardless of the precise chemical mechanisms, the data reported here demonstrate a clear-cut difference between *Eco*RV and *Eco*RI. It has been suggested that type II restriction enzymes can be categorized into two main groups, one typified by *Eco*RV and *Taq*I and another by *Eco*RI and *Bam*HI (Zebala et al., 1992; Newman et al., 1994). However, the recent structure determinations of *Bam*HI and *Pvu*II have revealed that both of these enzymes have three carboxylic acids at their active sites (Newman et al., 1994; Cheng et al., 1994), thus raising the possibility that they may have in common with *Eco*RV a two-metal mechanism rather than the one-metal scheme for *Eco*RI. The effect of Ca^{2+} on the activity of a new restriction enzyme might provide a simple test for its categorization.

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