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Asp-59 is not important for the catalytic activity of the restriction endonuclease *Eco*RI

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Abstract The amino acid Asp-59 was proposed to be involved in *Eco*RI catalyzed DNA cleavage (Cheng et al., EMBO J. 13, 3927–35, 1994). We have tested this hypothesis by site directed mutagenesis experiments. The four mutants D59A, D59E, D59G, and D59N bind with similar stability to the specific recognition sequence as wild type *Eco*RI. The D59E mutant cleaves DNA as fast as the wild type enzyme. Specific activities of the other three mutants are five to tenfold lower. Therefore, we conclude that Asp-59 is not involved in catalysis of the *Eco*RI restriction endonuclease. Consequences for catalytic mechanisms of *Eco*RI and other restriction enzymes are discussed.

Key words: Restriction enzyme; Catalysis; Protein–DNA interaction; Mg^{2+} coordination

1. Introduction

With the exception of some isoschizomers type II restriction endonucleases are very heterogeneous in their amino acid sequences [1]. On the other hand, the known three dimensional structures of four restriction enzymes, namely *Eco*RI [2], *Eco*RV, *Pvu*II [4,5], and *Bam*HI [6,7], show striking homologies. The overall folds of *Eco*RI and *Bam*HI as well as parts of the folds of *Eco*RV and *Pvu*II are similar [8]. All four enzymes have a central five stranded ß-sheet which harbours the catalytic centre composed of three homologous amino acid residues [9]. In the cocrystal structures these residues (Asp-91, Glu-111 and Lys-113 in *Eco*RI, Asp-74, Asp-90 and Lys-92 in *Eco*RV, Asp-58, Glu-68 and Lys-70 in *Pvu*II and Asp-94, Glu-111 and Glu-113 in *Bam*HI) are in a similar position relative to the phosphodiester bond to be cleaved (Fig. 1) and their importance for catalysis was verified by site directed mutagenesis experiments for *Eco*RI [10–13], *Eco*RV [14] and *Bam*HI [15,16]. The similar architecture of the catalytic centres of the four enzymes suggests that they use the same catalytic mechanism for DNA cleavage.

Based on molecular modelling for *Eco*RI and *Eco*RV we have proposed a mechanism of substrate assisted catalysis [17] with the following features: (i) the phosphate next to the phosphodiester bond to be cleaved abstracts a proton from an attacking water molecule, (ii) one Mg^{2+} ion positioned by two acidic residues of the active centre polarizes the phosphate to be attacked, (iii) a basic residue of the catalytic centre neutralizes the extra negative charge of the pentacoordinated transition state, and (iv) a water molecule of the hydration shell of the Mg^{2+} ion protonates the leaving group. In support of this model, we could show that the charge of the activating phosphate is essential for the catalytic activity of *Eco*RI and *Eco*RV [18] as it is for several other restriction endonucleases including *Bam*HI and *Pvu*II [19].

For *Eco*RV an alternative catalytic mechanism was proposed [20,21] in which two Mg^{2+} ions are involved, the main mechanistic difference being that one of the Mg^{2+} ions activates the attacking water molecule. To bind two divalent metal ions at least a third acidic amino acid in the catalytic centre is necessary. According to the X-ray structure analysis of several *Eco*RV-DNA-cocrystals one metal binding site could be formed by Asp-74 and Asp-90 and the second one by Glu-45 and Asp-74 [22]. In *Pvu*II also a third acidic residue (Glu-55) is close to the catalytic centre [1]. Its involvement in catalysis, however, has not been studied so far. In *Bam*HI the situation becomes more complicated due to the finding that four acidic residues of this enzyme are essential for catalysis [15,16]. These include (i) Asp-94 and Glu-111 which are homologous to the two acidic residues in the catalytic centres of the three other restriction enzymes, i.e. for example Asp-74 and Asp-90 in *Eco*RV (Fig. 1), (ii) Glu-77 which is a little distant from the catalytic centre and may be involved in binding the second metal ion like Glu45 in *Eco*RV, and (iii) Glu-113 which is at the position of the homologous lysine residues in the three other enzymes. For the formulation of a unified mechanism for all four enzymes this residue poses some difficulties because it is as essential for *Bam*HI as the lysine residues are for *Eco*RI [13] and *Eco*RV [14]. Comparison of the four X-ray structures led Cheng et al. [5] to the suggestion that Asp-59 in *Eco*RI may be involved in catalysis, analogous to Glu-77 in *Bam*HI, Glu-45 in *Eco*RV, and possibly Glu-55 in *Pvu*II. This would be in favour of a two metal ion mechanism for all four enzymes. Therefore, we decided to investigate the importance of Asp-59 for *Eco*RI catalyzed DNA cleavage by site directed mutagenesis.

2. Materials and methods

2.1. Mutagenesis and purification

Site directed mutagenesis was carried out according to the gapped duplex method as described by Geiger et al. [23]. We mutated a modified *ecoRI* gene which codes for an enzyme with a C-terminal His_6-tag. This allowed a simple and fast purification procedure by affinity chromatography. This modification reduces the specific activity only slightly by a factor of three [24]. The following set of *Eco*RI mutants at codon 59 was generated: Asp-59→Ala (D59A), Glu (D59E), Gly (D59G), and Asn (D59N). The mutations were verified by sequencing one strand of the entire *ecoRI* gene using four different primers which give overlapping sequencing ladders.

In order to characterize their enzymatic properties mutant proteins were purified by affinity chromatography. For this purpose cells of induced 100 ml cultures of the *E. coli* strain TGE900 (su^-, ilv^-, bio[λcI857, ΔBamΔHI], F^-, (pEcoR4)) that carried the expression vectors with the mutant gene were collected and resuspended in a buffer containing 30 mM KP_i pH 7.2/0.1 M DTE/0.01% Lubrol (PDL) and 500 mM NaCl. All subsequent steps were carried out at

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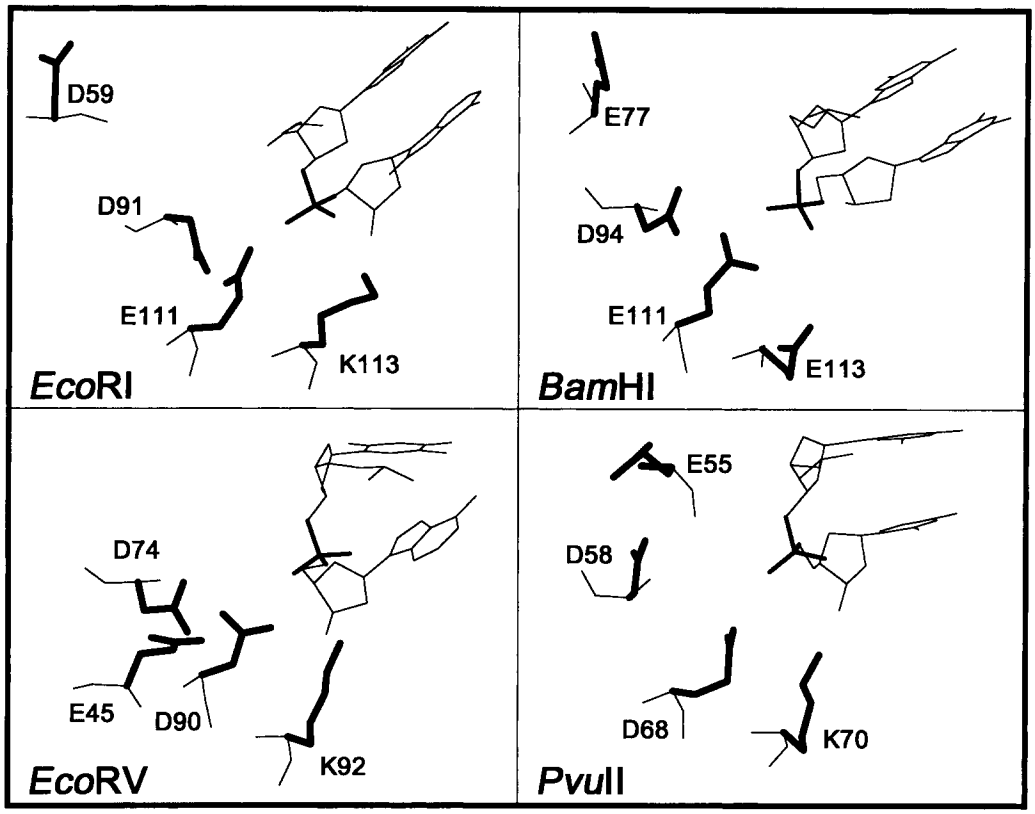

Fig. 1. Structures of the catalytic centres of *Eco*RI (upper left, from 1ERI in the Brookhaven data bank), *Bam*HI (upper right, coordinates from Dr. A.K. Aggarwal), *Eco*RV (lower left, 1RVA) and *Pvu*II (lower right, 1PVI). The positions of two acidic (e.g. Asp-91 and Glu-111 in *Eco*RI) and one basic residue (e.g. Lys-113 in *Eco*RI but Glu-113 in *Bam*HI) are nearly identical, while a further acidic residue (Asp-59 in *Eco*RI, Glu-77 in *Bam*HI, Glu-45 in *Eco*RV and Glu-55 in *Pvu*II) is at different locations in the four enzymes.

4°C. Following disintegration of the cells by sonication, the His_6-tagged enzymes were bound to 1 ml Ni^{2+}-NTA-agarose (Qiagen) as affinity matrix. Protein loaded columns were subsequently washed with 10 ml PDL pH 7.5 / 500 mM NaCl / 10 mM imidazole and 10 ml PDL pH 7.5/500 mM NaCl/50 mM imidazole. The proteins were eluted with 1.5 ml PDL pH 7.5/500 mM NaCl/150 mM imidazole. The resulting preparations were >90% pure on denaturing gel electrophoresis. Protein concentration was determined spectrophotometrically [23]. To eliminate any influence of the His_6-tag or of the short purification protocol on the results wild type His_6-tagged *Eco*RI was purified in parallel by the same procedure and used as a control in all experiments described here.

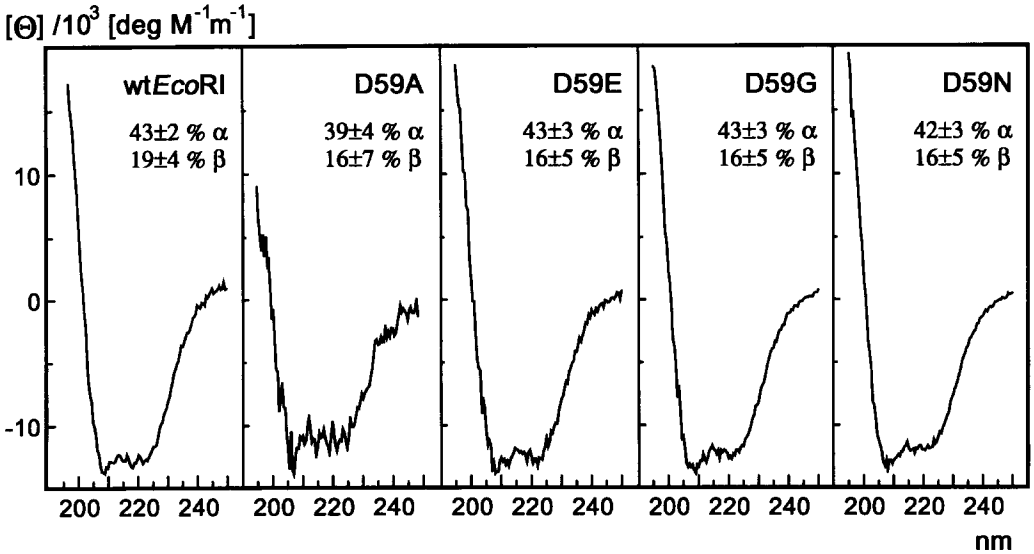

Fig. 2. Circular dichroism spectra recorded with 0.19 mg/ml wild type *Eco*RI (two spectra accumulated), 0.08 mg/ml D59A (three spectra accumulated), 0.26 mg/ml D59E, 0.35 mg/ml D59G and 0.4 mg/ml D59N in 20 mM Tris-HCl pH 7.5/1 mM EDTA/300 mM NaCl. The secondary structure contents indicated were calculated according to Chen et al. [35].

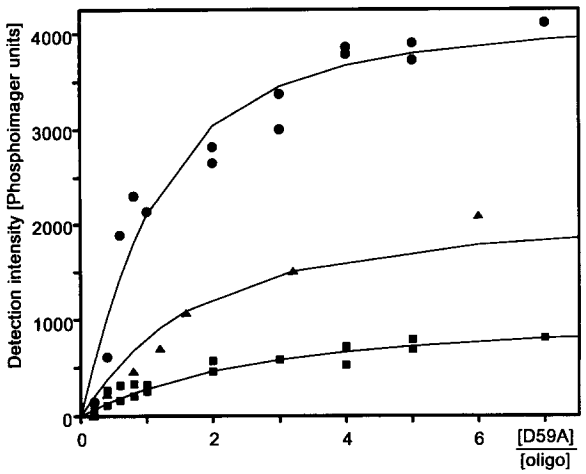

Fig. 3. Nitrocellulose filter binding results for the D59A mutant complexed with the self complementary oligodeoxynucleotide d(TATAGAATTCTAT) (■: 2.5 nM, ▲: 5 nM, ●: 10 nM). The curves represent the best fit simultaneously to all data points [23] with a specific binding constant of $1.9 \pm 0.6 \times 10^8$ M^{-1}. Similarly binding constants of $1.2 \pm 0.3 \times 10^8$ M^{-1} for D59E, $1.3 \pm 0.4 \times 10^8$ M^{-1} for D59G, and $1.3 \pm 0.4 \times 10^8$ M^{-1} for D59N were obtained. The determined specific binding constant of wild type *Eco*RI was $9.3 \pm 0.7 \times 10^7$ M^{-1}.

2.2. Circular dichroism CD spectroscopy

Parts of the enzyme preparations were dialyzed against storage buffer (PDL pH 7.5/300 mM NaCl/70% glycerol v/v) (i) to remove imidazole which interferes with circular dichroism measurements and (ii) to concentrate the enzymes about fourfold. The resulting solutions were directly analyzed using a cuvette with a pathlength of 0.1 cm in a Jobin Yvon Dichrograph III with a bandwidth of 2 nm, a rate of scanning of 0.03 nm and a time constant of 2 s as described [23].

2.3. DNA binding experiments

Specific DNA binding was measured in nitrocellulose filter binding experiments using the self complementary oligodeoxynucleotide d(TATAGAATTCTAT) as described [25]. Quantitation of radioactive spots on the filters was performed by using a Fuji BAS 1000 bio imaging analyzer 486 PC.

2.4. DNA cleavage experiments

In order to determine the cleavage activities of the mutants and the wild type enzyme bacteriophage λ DNA was used as substrate. 0.25 μg/15 μl of the λ DNA was incubated with 1 nM enzyme at 37°C in a buffer containing 20 mM Tris-HCl pH 7.5/50 mM NaCl/10 mM $MgCl_2$. Aliquots were taken at different time points, the reaction was stopped by adding agarose gel loading buffer and analyzed by electrophoresis using 0.8% agarose gels. Ethidium bromide stained gels were documented using the E.A.S.Y. store video system (Herolab GmbH Laborgeräte, Wiesloch, Germany). Specific activity was calculated from the time point at which the complete cleavage pattern was detected and, therefore, is accurate within a factor of 2–3.

3. Results and discussion

In addition to the known amino acid residues of the catalytic centre of the restriction endonuclease *Eco*RI (Asp-91, Glu-111 and Lys-113) a third acidic residue, Asp-59, was proposed to play a role in catalysis [5]. To check this proposal we have mutated this residue to alanine, glutamic acid, glycine and asparagine. Mutagenesis was carried out with a His_6-tagged version of the *Eco*RI endonuclease in order to allow for a fast one step purification by affinity chromatography. The enzymes were overproduced and then purified by Ni^{2+}-NTA-agarose chromatography.

In order to detect possible changes in secondary structure composition of the mutant enzymes we determined their CD. The spectra of the D59E, D59G and D59N mutants are very similar to that of the wild type *Eco*RI (Fig. 2). The differences in the fitted secondary structure content are within the limits of error. The D59A mutant has a slightly lower secondary structure content. But this enzyme had the lowest solubility and as can be seen in Fig. 2 had the lowest signal to noise ratio of all the proteins studied. A change in secondary structure composition would be rather anticipated for the D59G than the D59A mutant. The good binding and cleavage activities of D59A (see below) argue furthermore against a remarkable structural change. Therefore, we suggest that at least under cleavage conditions or in a complex with its specific DNA sequence this mutant retains essentially the same structure as *Eco*RI and the other three mutants.

Binding to the specific recognition sequence was measured using the nitrocellulose filter binding technique. Binding for the His_6-tagged enzymes including wild type *Eco*RI differs only within a factor of two. The mutants bind the specific substrate even slightly better than the wild type enzyme (Fig. 3). Therefore, the negative charge at position 59 is not important for specific binding. This finding was not unexpected, since the binding specificity is mainly caused by direct contacts of amino acid residues of the extended chain motif to the bases of the recognition sequence [26,27]. This sequence motif is very distant from Asp-59 both in the primary and tertiary structures. Relatively close in the tertiary structure to Asp-59 are, however, three amino acid residues which make contacts to flanking phosphates. Our data show no indication that these contacts are influenced by the Asp-59 mutants. The absence of any effect on binding strength is typical for catalytic site mutations, they should, however, impair DNA cleavage.

The specific activity of the enzymes was measured using λ DNA as substrate. The D59E mutant has exactly the same specific activity as wild type *Eco*RI ($1.1 \pm 0.5 \times 10^6$ U/mg,

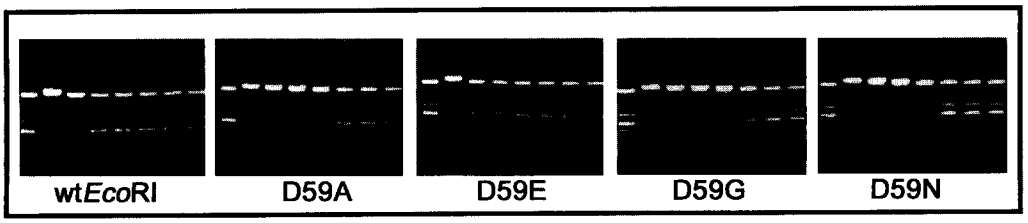

Fig. 4. Ethidium bromide stained 0.8% agarose gels showing the λ DNA cleavage patterns of wild type *Eco*RI and Asp-59 mutants. 1 nM enzyme was incubated with 2 μg λ DNA/120 μl at 37°C. Aliquots were taken at 1, 8, 15, 30, 60, 90, and 120 min. A standard with the complete canonical cleavage pattern is shown in each first lane.

Fig. 4). The three other mutants are about 10 fold less active but still cleave DNA (D59A and D59N $1.8 \pm 0.5 \times 10^5$ U/mg, D59G $1.3 \pm 0.3 \times 10^5$ U/mg). Therefore, an acidic amino acid at position 59 is favourable for *Eco*RI activity but not necessary. Glutamic acid can fully substitute for aspartic acid at position 59. Furthermore, the adequate activity of the other three mutants strongly argues against an essential role for Asp-59 in catalysis, whereas *Eco*RI proteins with mutations of amino acid residues Asp-91, Glu-111 and Lys-113 which were confirmed to be involved in catalysis are at least a thousandfold less active than the wild type enzyme [10–13].

The question remains whether there are other acidic residues in the *Eco*RI structure which could be involved in binding of a second metal ion (in the catalytic centre). Only Glu-144 as a possible ligand is as close as Asp-59 to the catalytic centre. In the crystal structure this residue is embedded in a hydrogen bonding network connecting base contacting residues of both subunits [26]. This important although indirect role in sequence recognition is reflected in the results of extensive mutagenesis studies [10,28–30] which gave no indication of an involvement of Glu-144 in catalysis. Therefore, we conclude that the catalytic centre of *Eco*RI is composed of one basic (Lys-113) and only two acidic residues (Asp-91 and Glu-111). This rules out a two metal ion mechanism for *Eco*RI.

For a unified catalytic mechanism of restriction endonucleases one has to discuss the experimental evidences for the two mechanisms proposed also with respect to the other enzymes. Mainly two lines of evidence led to the proposal of a two metal ion mechanism for *Eco*RV catalyzed DNA cleavage: (i) In several crystals soaked with Mg^{2+} or other divalent cations electron density interpreted to be due to two divalent metal ions was found close to the scissile phosphodiester bond [22]. One site of higher electron density was clearly seen with several divalent cations between Asp-74, Asp-90 and the attacked phosphate; these two acidic residues had been shown by site directed mutagenesis experiments to be essential for catalysis (the D74A, N or D90A, N mutants do not cleave DNA at all) [14]. A second electron density at Glu-45 and Asp-74 is only seen with certain metal ions [22]. It was clearly found in an *Eco*RV product crystal after substrate cleavage. Site directed mutagenesis experiments indicated that Glu-45 was not as important for catalysis as the other two acidic residues. Although cleavage activity of the E45A mutant is 10.000 fold reduced that of the E45Q mutant is only 300 fold lower than wild type activity [13]. (ii) Low concentrations of Ca^{2+} stimulate the Mg^{2+} or Mn^{2+} dependent DNA cleavage of *Eco*RV although Ca^{2+} alone does not lead to cleavage. Therefore, it was concluded that for this enzyme a second metal binding site is important for catalysis [20]. These observations, however, do not allow an assignment of the site to the catalytic centre. In contrast to *Eco*RI divalent metal ions are not only necessary for *Eco*RV catalyzed cleavage but also for sequence specific binding [31,32]. Ca^{2+} can promote sequence specific binding but not DNA cleavage [33]. This holds even for a triple mutant lacking all three acidic residues (Glu-45, Asp-74 and Asp-90) involved in metal binding according to the X-ray structure [34]. The metal binding site required for sequence specific DNA binding was located next to the phosphate two nucleotides 5′ of the scissile bond, thus far away from the catalytic centre. Therefore, in *Eco*RV three binding sites for divalent cations have been proposed promoting two essential roles, i.e. site specific DNA binding and cleavage. These findings are still compatible with both mechanisms. In favour of substrate assisted catalysis is the requirement of a negative charge at the activating phosphate which is not participating in the two metal ion mechanism [18]. From two cocrystal structures with different oligodeoxynucleotide substrates, however, only one has a favourable distance between the attacked and the water activating phosphate [3,22]. The DNA in this cocrystal was cleaved after soaking with Mg^{2+} whereas in the other cocrystal with an unfavourable geometry the DNA stayed intact. All these experimental data present substantial evidence for substrate assisted catalysis also in the case of *Eco*RV.

The recent cocrystal structure of *Bam*HI with a cognate oligodeoxynucleotide [7] is compatible with both mechanisms. As already described the catalytic centre is formed by three acidic residues because Glu-113 replaces the lysine residue found in the three other enzymes. Three water molecules can be seen at almost identical positions in the catalytic centres of both independently solved subunits of the dimeric enzyme. Two of them are at homologous positions to the two metal ions in the exonucleolytic domain of the Klenow polymerase and the third is hydrogen-bonded to the phosphate of the neighbouring 3′ nucleotide and almost in the position of an in line attack. In *Bam*HI two divalent metal ion binding sites have been clearly assigned to the catalytic centre. In this case the presence of two metal ions is required for both mechanisms under discussion. The first metal ion polarizes the phosphate to be attacked, stabilizes the transition state and leads to protonation of the leaving group in both mechanistic models. The second metal ion activates the attacking water molecule (two metal ion mechanism) or neutralizes the extra negative charge of the transition state (substrate assisted catalysis). In the latter case Glu-113 and its bound Mg^{2+} ion mimic a lysine residue. Unfortunately the situation is not as easy because the E113K mutant of *Bam*HI is as inactive as the corresponding mutants K113E of *Eco*RI and K92E of *Eco*RV [16,13,14]. K92E, however, can give a hint for resolving the complicated situation. It is active in the presence of Mn^{2+} which is one of the divalent metal ions responsible for the electron density in the second metal ion binding site in the *Eco*RV structure. We interprete this in the following way: the catalytic centre of *Eco*RI is too narrow to accomodate a second divalent cation even if the lysine residue is mutated to glutamic acid. In *Eco*RV the observed weak second binding site becomes particularly relevant for the K92E mutant in combination with Mn^{2+} while in *Bam*HI it is necessary and structural constraints do not allow a substitution by lysine. Therefore, two metal binding sites in *Bam*HI do not necessarily imply the two metal ion mechanism which we can rule out for *Eco*RI.

From these data we conclude that substrate assisted catalysis is operative for *Eco*RI and we favour it also for the three other restriction enzymes where structural information is available. The essentiality of the negative charge at the activating phosphate was shown for all of them [18,19]. Additional structural details about metal binding sites in *Bam*HI and *Pvu*II and mutagenesis results in *Pvu*II will further help to decide whether all four enzymes use essentially the same catalytic mechanism.

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