J. Biochem. Mol. Biol. Vol. 29, No. 1 pp. 17~21 January 31, 1996

Site-Directed Mutagenesis of Ile91 of Restriction Endonuclease *Eco*RV: Dramatic Consequences on the Activity and the Properties of the Enzyme

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(Received August 10, 1995)

Abstract: Ile91 of restriction endonuclease *Eco*RV, which has not been known to take part directly in catalytic activity, was substituted with Leu by site-directed mutagenesis. The Ile91Leu mutant shows over 1000-fold less activity than the wild type *Eco*RV under standard reaction condition. The metal ion dependency of the reaction was altered. In contrast to the wild type *Eco*RV, the mutant prefers Mn^{2+} to Mg^{2+} as the cofactor. In Mn^{2+} buffer the mutant is as active as the wild type enzyme in Mg^{2+} buffer. Like the wild type enzyme, the mutant shows an unspecific binding of DNA in gel shift experiments. In contrast to the wild type enzyme, the mutant did not cleave at noncognate sites of DNA under star condition.
Key words: restriction endonuclease *Eco*RV, site-directed mutagenesis.

*Eco*RV is a type II restriction endonuclease (Bennet and Halford, 1989). It recognizes and cleaves DNA at the GATATC sequence, leaving blunt-ended fragments (Kholmina *et al.*, 1980). *Eco*RV exists as a dimer of 2 identical subunits and it requires Mg^{2+} for cleavage. The structures of the free protein bound to both specific and non-specific DNA molecules have been solved by X-ray crystallography (Winkler, 1992). The complexes were crystallized in the absence of Mg^{2+} which is an essential cofactor for DNA cleavage conditions. From the crystal structures obtained, it was seen that the DNA in the specific complexes lies in the cleft between the 2 subuints of the dimer with its minor groove facing the bottom of the cleft and major groove facing the entrance. One peptide loop centered on Asn 70 approaches the minor groove, and all its interactions with the DNA are with the phosphates rather than the bases. Another loop centered on Asn185 penetrates deep into the major groove and interacts with the bases. The amino acids located in these two loop regions are responsible for DNA recognition (Theiking *et al.*, 1991). The crystallographic analysis has revealed the presence of a cluster of 3 acidic amino acids (Glu45, Asp74 and Asp90) and one basic amino acid (Lys92) in close proximity to the scissile phosphodiester bond. When *Eco*RV binds to the specific DNA, the DNA distorts and the conformation of the protein changes (Winkler, 1992). This brings the amino acids in the catalytic center close to the scissile phosphodiester bond. Asp74 and Asp90 are now oriented in a position favorable for binding Mg^{2+}.

Mg^{2+} polarizes the scissile P-O bond, enhancing its susceptibility to nucleophilic attack. The hypothesis put forward by Selent and his coworkers suggests that an acidic amino acid (either Asp74 or Asp90) activates a water molecule which then attacks the scissile bond. Mg^{2+} may be involved in binding the attacking water molecule. The extra negative charge of the pentacovalent phosphorus formed upon attack by the water molecule is compensated for by Lys92. Lys92 therefore serves to stabilize the transition state. The hydrolytic reaction proceeds with inversion of the configuration around phosphorous (Grasy and Connolly, 1992). The hypothesis accounts for why *Eco*RV binds all DNA sequences (both specific and non-specific) with equal affinity in the absence of Mg^{2+}, but cleaves its recognition site faster than other DNA sequences when Mg^{2+} is present. There is coupling between recognition and cleavage via formation of a Mg^{2+} binding site.

Random mutagenesis studies (Vipond *et al.*, 1994) on the region of *Eco*RV (residues 86-95) have found that the substitution of Ile91 by Leu leads to enzyme activity being decreased a thousand fold compared to the wild type. Leu91 does not take part directly in catalytic activity. In order to explain its role in catalysis,

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we attempted to change this residue by site-directed mutagenesis and analyze the effect of this replacement on the activity of *Eco*RV.

Materials and Methods

DNA manipulations

General manipulations of DNA were carried out as in Sambrook *et al.* (1989). DNA sequencing was by the dideoxy method (Sanger *et al.*, 1977). Oligonucleotide was synthesized at the Department of Biochemistry, University of Bristol, UK). Restriction enzymes and other DNA modifying enzymes were purchased and used as advised by the supplier. *Eco*RV was purified here.

Mutagenesis

Site-directed mutagenesis was performed by the modification of the Amersham mutagenesis system (code-RPN1523), which is based on the phosphorothioate method of Eckstein and his co-workers (Taylor *et al.*, 1985). The single strand template pRV18 was used. pRV18 is a derivative of pBluescript (Stratagene) carrying the *Eco*RV gene with a 30 base pair stuffer fragment to inactivate the gene (Vermote *et al.*, 1992). Positive clones were screened by restriction enzyme analysis and verified by sequencing the entire gene.

Enzyme overproduction and purification

The derivative of pRV18 coding for the Ile91Leu mutant of *Eco*RV was used to transform *E. coli* CSH50 carrying pMetB. The transformant was grown in L-broth at 55℃ to an OD_{600} of 0.4. An equal volume of L-broth at 55℃ was added and the growth continued for 4 h at 42℃. The cell was harvested by centrifugation and stored at −20℃. The cells were resuspended and disrupted by sonication, and the mutant *Eco*RV enzyme was purified by chromatography, first on phosphocellulose and then on Blue-Sepharose, as described previously with the wild type enzyme (Luke *et al.*, 1987). The purifications were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; the gels were developed with Coomasive Brilliant Blue-R-250. Protein concentrations were determined by the method of Bradford (1976) after calibration of the method by amino acid analysis. Concentrations of both the wild type and mutant *Eco*RV enzymes are given in terms of protein dimers of M_r 57000.

DNA binding

The binding of the wild type and mutant *Eco*RV enzymes to DNA was measured by gel-shift assays, as described by Taylor *et al.* (1991). The binding buffer was 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/ml BSA, and 0.1 mM EDTA, pH 7.5.

DNA cleavage

The substrate was the monomeric forms of pAT153 (Twigg and Sherratt, 1980), purified from transformants of *E. coli* HB101 that had been grown in M9 minimal salts with 1 mCi/l[methyl-^{3}H]thymidine (Halford and Goodall, 1988). The reactions with enzyme were carried out at 20℃ in buffer A (standard reaction condition) or in buffer B, with either $MgCl_2$ or $MnCl_2$ at the concentrations specified. Buffer A is 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, pH 7.5. Buffer B is 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, 10% (v/v) DMSO, pH 8.5. During each reaction, the changes in the concentrations of the supercoiled, open-circle, and linear forms of the DNA were measured as described previously (Halford and Goodall, 1988; Taylor and Halford, 1989).

Results and Discussion

Mutagenesis

The mutant oligonucleotide is designed such that the third base in the codon on the coding strand coding for Ile89 is changed, destroying the *Cla*I site (ATCGAT) but Ile89 is retained owing to the degenerate nature of the genetic code. This allowed us to screen for cells that have been transformed by mutant DNA through a simple restriction analysis with *Cla*I. Absence of this cleavable *Cla*I site would mean that the cells have been transformed with mutant DNA. Substituting the first base of the codon for residue 91 with C gave a leucine residue. There are 2 *Cla*I sites in the *Eco*RV cassette used. During mutagenesis a *Cla*I site was mutated and the absence of this cleavable *Cla*I site meant that the cell had been successfully transformed with mutated DNA. Positive clones were selected by *Cla*I restriction analysis. Within the *Eco*RV cassette we used there is a stuffer fragment containing 6 stop codons to prevent overexpression of the protein which would otherwise kill the cells. It was removed using *Pst*I, and the fragments ligated back. There is an *Eco*RV site present in the stuffer fragment and *Eco*RV was added then to select for molecules that have religated back without the stuffer fragment. The colonies from activated clones were grown overnight and the cultures were then inoculated into fresh medium, grown until turbid and later induced to overexpress the *Eco*RV mutant.

Purification of he Ile91Leu mutant

The purification of mutant protein from the cell lysa-

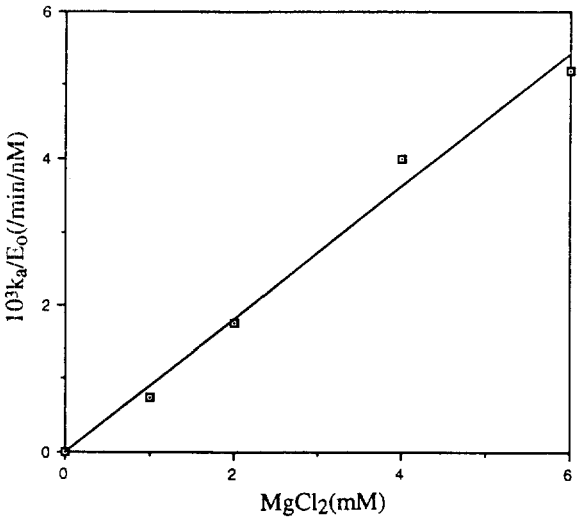

Fig. 1. Magnesium concentration dependencies of Ile91Leu *Eco*RV mutant. For each reaction, a first-order rate constant (ka) was evaluated from the decline in the amount of supercoiled DNA. The rate constants were normalized against the concentration of the enzyme, to produce values for ka/[Eo].

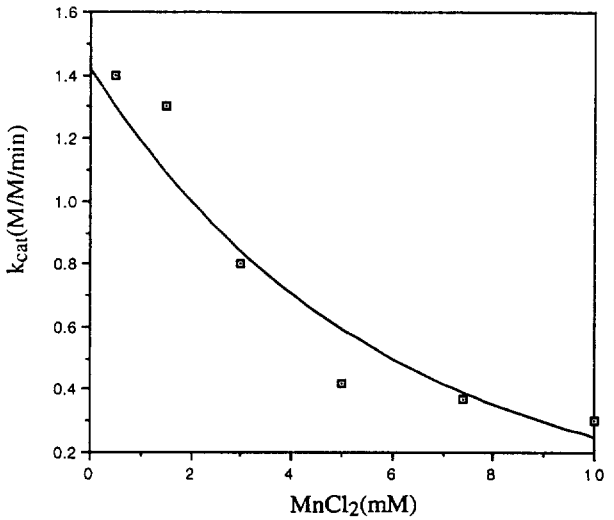

Fig. 2. Manganese concentration dependencies of Ile91Leu *Eco*RV mutant Values for kcat were determined from the steady-state reaction velocities.

tes was carried out essentially as described previously with the wild type enzyme (Luke *et al.*, 1987). The purifications of the protein by chromatography on phosphocellulose gave reasonably pure protein by polyacryl-amide gel electrophoresis.

Further purification by affinity chromatography on Blue-Sepharose afforded a homogeneous protein. SDS-polyacryl-amide gel electrophoresis shows a single band. DNA cleavage Experiments with the Ile91Leu Mutant . The DNA cleavage activity of the mutant was determined with pAT153 as a substrate in the presence of Mg^{2+} or Mn^{2+}, respectively. Under standard condition (see Material and Methods), the mutant shows over 1000-fold less activity than wild type *Eco*RV. The Mg^{2+} dependency of the reaction was also altered (Fig. 1). In contrast to the wild-type where the reaction rates were invariant with increasing concentration of $MgCl_2$ (Halford and Goodall, 1988), the activity of the mutant enzyme increased with the magnesium ion concentration, indicating that, the mutant was not fully saturated with the metal ion over the range studied. The Ile to Leu substitution has somehow perturbed the metal binding site of the enzyme, such that the affinity of the enzyme when bound to GATATC has been reduced. The wild type enzyme is more active with Mg^{2+} as the cofactor than with Mn^{2+}. In contrast, experiments with Mn^{2+} buffer revealed that the mutant prefers Mn^{2+} as the cofactor. Under these conditions, the mutant is as active as the wild type enzyme in Mg^{2+} buffers. However, the Mn^{2+} activity profile is unusual, revealing a decrease in activity with increasing concentration of Mn^{2+} (Fig. 2). In the reaction with wild type *Eco*RV, no variation in kcat was observed as the concentration of $MnCl_2$ was varied from 1 to 10 mM from an activity of zero in the absence of $MnCl_2$, it increased to its maximum at the lowest concentration of $MnCl_2$ tested (0.5 mM), but further increases in the concentration of Mn^{2+} then caused a progressive reduction in activity. The decrease in kcat as more $MnCl_2$ was added to the reactions cannot be due to an increase in ionic strength, because reactions were at a constant ionic strength, maintained by adjusting the concentration of NaCl. One possible explanation of this phenomena is the presence of two metal binding sites in the enzyme's active site (Vipond *et al.*, 1995), and that the mutant has altered the geometry of the enzyme in a way that causes binding of the second metal ion to disrupt the active conformation. The active site of *Eco*RV contains two potential sites for binding divalent metals: one between Asp90 and Asp74, and another between Asp74 and Glu45.

DNA binding by the Ile91Leu *Eco*RV mutant

The binding properties of the Ile19Leu mutant to DNA were compared with those of the wild type *Eco*RV (Fig. 3). The binding studies were carried out by the gel shift method (Garner and Revzin, 1981; Taylor *et al.*, 1991), under conditions where the DNA cannot be cleaved due to the absence of divalent ions. The gel shift method is based on DNA-protein complexes having different electrophoretic mobilities from the free DNA. It can be used to separate the free DNA from DNA-protein complexes, but complexes with different protein stoichiometries on the same DNA can also be separated from each other (Fried, 1989). Consequently, if the low activity of the mutant is caused

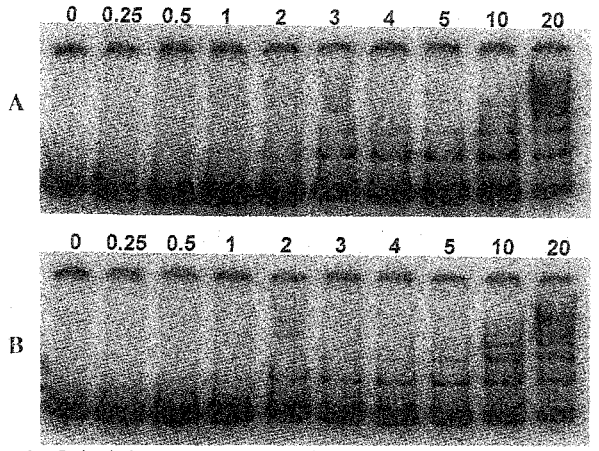

Fig. 3. Gel shift experiments with *Eco*RV-DNA complexes (A) and Ile91Leu mutant-DNA complexes (B). The reactions contained the restriction endonucleases at concentrations (nM) indicated above each lane and approximately 0.1 nM ^{32}P-labeled DNA in binding buffer. Lane 0 is in the absence of the enzyme.

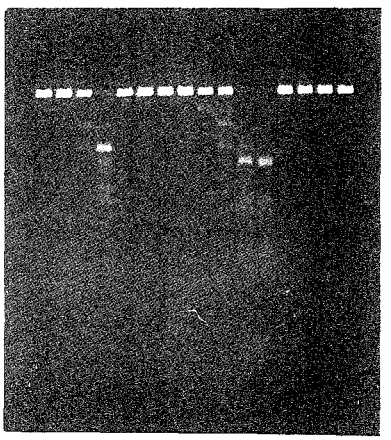

Fig. 4. Cleavage of noncognate sites of DNA by *Eco*RV and Ile91Leu mutant. Reactions in buffer B contained 10 nM DNA with Mg^{2+} (lane 1~8) or in buffer A contained Mn^{2+} (lane 9~16). The concentrations of *Eco*RV (lane 1~4 and 9~12) and the mutant (lane 5~8 and 13~16) were 1, 10, 100, 1000 nM, respectively. Reactions were initiated by the addition of the enzyme at 20℃ and after 20 min, the reaction was stopped. The top bands are initial products as full length linear form from supercoiled DNA. Lanes 3, 4, 9, 10, 11 and 12 show several smaller fragments produced from initial products.

by low affinity of the enzyme to DNA, gel shift analysis would show different pattern compared to that of wild type enzyme. The DNA used in the binding studies was the same 381 bp *Eco*RI-*Hin*dIII fragment from pAT153, ^{32}P-labeled at its 5′-ends, that had been used before for binding studies on wild type *Eco*RV (Taylor *et al.*, 1991; Thielking *et al.*, 1992). This fragment contains the *Eco*RV site from pAT153 and an additional 366 nonspecific site for a protein like *Eco*RV that covers about 15 bp, though only 25 or so of these sites can be occupied on each DNA molecule at any given time (Taylor *et al.*, 1991). Fig. 3 shows that the mutant yielded the same array of DNA-protein complexes as the wild type restriction enzyme: Like wild type *Eco*RV, the mutant shows unspecific binding. The mutant has the same binding pattern to DNA as wild type *Eco*RV.

DNA cleavage under star reaction conditions

The ability of restriction enzymes to discriminate between DNA sequences varies with the reaction condition (Polisky *et al.*, 1975; Tikchonenko *et al.*, 1978; Barrany, 1988). *Eco*RV shows relaxed specificity under a reaction condition which is high pH and low salt concentration and also in the presence of water-miscible organic solvents (Halford *et al.*, 1986; Bennett and Halford, 1989; Taylor and Halford, 1989). Buffers containing organic solvents such as dimethyl sulphoxide can enhance the activity at noncognate sites, without having much effect on cognate activity: these used to be known as "star conditions" (Halford *et al.*, 1986). The *Eco*RV mutant was tested for specificity under star condition with pAT153 as substrate. Plasmid pAT153 contains, in addition to one *Eco*RV recognition site, 12 noncognate sites that can be cleaved by *Eco*RV under star condition with a high concentration of the enzyme. Surprisingly, in contrast to the wild-type, the mutant did not cleave at noncognate sites under star condition with Mg^{2+} and Mn^{2+}. Even though we increased the concentration of the mutant from 1 nM to 1000 nM in buffer B with Mg^{2+} and Mn^{2+}, the mutant enzyme did not lose its specificity (Fig. 4).

Despite the availability of extensive X-ray crystallography data on *Eco*RV, both with and without specific and non-specific DNA (Winkler, 1992), the precise role of Ile91 can be only be guessed at. In crystal structures, the side chain of Ile91 points towards Glu45, so some subtle conformational packing at this metal binding site may be responsible for the characteristics of the mutated enzyme. These results suggest that minor alterations of the protein, at residues that have no direct role in either DNA recognition or catalysis, can thus have dramatic consequences on the activity and properties of the enzyme by perturbing the alignment of the functional groups that are essential for either DNA recognition or catalysis.

Acknowledgement

This work was supported by a grant to B. J. Moon from the Korea Science and Engineering Foundation through Center for Biofunctional Molecules and the British Council.

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