A Site-Directed Mutagenesis Study To Identify Amino Acid Residues Involved in the Catalytic Function of the Restriction Endonuclease *Eco*RV†

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ABSTRACT: We have used site-directed mutagenesis of the *Eco*RV restriction endonuclease to change amino acid side chains that have been shown crystallographically to be in close proximity to the scissile phosphodiester bond of the DNA substrate. DNA cleavage assays of the resulting mutant proteins indicate that the largest effects on nucleolytic activity result from substitution of Asp74, Asp90, and Lys92. We suggest on the basis of structural information, mutagenesis data, and analogies with other nucleases that Asp74 and Asp90 might be involved in Mg^{2+} binding and/or catalysis and that Lys92 probably stabilizes the pentacovalent phosphorus in the transition state. These amino acids are part of a sequence motif, Pro-Asp...Asp/Glu-X-Lys, which is also present in *Eco*RI. In both enzymes, it is located in a structurally similar context near the scissile phosphodiester bond. A preliminary mutational analysis with *Eco*RI indicates that this sequence motif is of similar functional importance for *Eco*RI and *Eco*RV. On the basis of these results, a proposal is made for the mechanism of DNA cleavage by *Eco*RV and *Eco*RI.

Considerable effort has been devoted to elucidate the mechanism by which type II restriction endonucleases recognize their target sequences [see reviews by Bennett and Halford (1989), Rosenberg (1991), Winkler (1992), and Heitman (1992)]. Comparatively few studies have addressed the question how phosphodiester bond cleavage is catalyzed after the process of recognition. Type II restriction enzymes catalyze the hydrolysis of specific phosphodiester bonds, generating 5′-phosphorylated DNA fragments with sticky ends (e.g., for *Eco*RI, G/AATTC) or blunt ends (e.g., for *Eco*RV, GAT/ATC). Both DNA strands are cleaved in individual reactions that can occur separately or in a concerted manner, depending on restriction enzyme, substrate, and reaction conditions. Mg^{2+} is an essential cofactor for DNA cleavage by restriction endonucleases. It can be substituted by some other divalent cations, albeit with a reduction in activity and/or specificity [see reviews by Modrich (1982) and Malcolm (1987)]. Because all restriction enzymes need Mg^{2+} for their hydrolytic action but not necessarily for specific binding, it is likely that Mg^{2+} is directly involved in catalysis. Its precise role, however, is uncertain. It may be involved in activating a water molecule, in making the phosphorus more electrophilic, in stabilizing the transition state, or in neutralizing the leaving group charge. It has been shown for *Eco*RI (Connolly et al., 1984) and *Eco*RV (Grasby & Connolly, 1992) that the hydrolytic reaction proceeds with inversion of configuration around phosphorus. This result is in agreement with an enzyme-catalyzed direct nucleophilic attack of H_2O at phosphorus without involvement of a covalent enzyme intermediate. However, the mechanism of phosphodiester bond cleavage and the amino acid residues that are responsible for catalysis and/or Mg^{2+} binding are not yet known for any restriction enzyme.

The structures of two restriction enzyme–DNA complexes have been solved by X-ray crystallography (Kim et al., 1990; Rosenberg, 1991; Winkler et al., 1992; Winkler, 1992). Both the *Eco*RI–DNA and *Eco*RV–DNA complexes were crystallized in the absence of Mg^{2+}. Therefore, the structure analyses do not provide direct information about the location of the catalytic center. For both enzymes, however, the amino acid residues in the vicinity of the scissile phosphodiester bonds can be identified. Furthermore, in co-crystals soaked with Mn^{2+}, the amino acid residues in proximity to the metal ion have been determined (Rosenberg, 1991; Winkler et al., 1992). This information allows us to approximately locate the catalytic sites of the two enzymes in their three-dimensional structures, namely, around Glu111 in *Eco*RI and around Asp90 in *Eco*RV.

Independent evidence for this identification can be derived from the comparison of the *Eco*RI and *Eco*RV structures, which has revealed a structural homology between the two enzymes. In *Eco*RI, Glu111 is part of a β-strand running parallel to the DNA backbone in the *Eco*RI–DNA complex. Nearby, there is Lys113, and vis à vis as part of a loop, there are Pro90 and Asp91. In *Eco*RV, homologous residues in a structurally similar location are found, namely, Asp90 and Lys92 in a β-strand parallel to the DNA backbone in the *Eco*RV–DNA complex and Pro73 and Asp74 in a nearby loop (Thielking et al., 1991). The *structural* homology in this critical region suggests a *functional* homology, in as much as these two enzymes may make use of the same basic mechanism to accomplish phosphodiester bond cleavage (Figure 1). This assumption is supported by the sequence comparison between *Eco*RI and its isoschizomer *Rsr*I which have identical amino acids at these positions while on average the homology is 50% (Stephenson et al., 1989).

In order to test the functional importance of amino acid residues located in the vicinity of the scissile phosphodiester bond in the *Eco*RV–DNA complex (Winkler et al., 1992), we changed these residues by site-directed mutagenesis and analyzed the effects of these replacements on the activity of *Eco*RV. Furthermore, to demonstrate the similar function of

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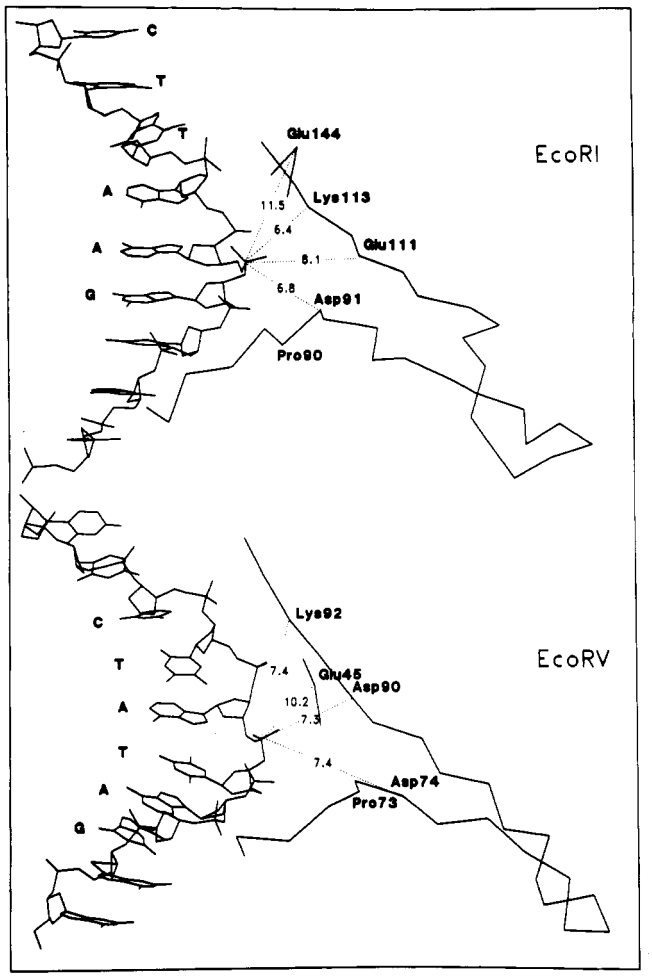

FIGURE 1: Presumptive active centers of *Eco*RI and *Eco*RV. The figure depicts part of the structure of the *Eco*RV–DNA (bottom) and the *Eco*RI–DNA complex (top) using the coordinates deposited in the Brookhaven protein data bank. For clarity, only one strand of the DNA is shown. The polypeptide backbone is represented by the C_α positions. Indicated are the C_α–P distances between several residues of *Eco*RI and *Eco*RV, respectively, and the scissile phosphodiester bond.

those amino acid residues in *Eco*RI, which are in a structurally analogous location as in *Eco*RV, we have also carried out a mutational analysis of some of these residues. The results of this analysis have allowed us to confirm the tentative assignment of the active sites of *Eco*RV and *Eco*RI and to put forward a proposal for the mechanism of DNA cleavage by these two restriction enzymes.

Experimental Procedures

Bacterial Strains and Plasmid Vectors. The following *Escherichia coli* strains were used: WK6mutS(λ) for transformation of DNA after mutagenesis; LK111(λ) for propagation of DNA; TGE900 and 5112 [pRK248cIts] for the overexpression of *Eco*RI and *Eco*RV, respectively (Geiger et al., 1989; Thielking et al., 1991). Prior to transformation with the plasmids harboring the *Eco*RI or *Eco*RV gene, all strains were transformed either with plasmid pECOR4 (Bougueleret, 1985) carrying the *Eco*RI methyltransferase gene or with pLBM4422 (Thielking et al., 1991) carrying the *Eco*RV methyltransferase gene, because for overexpression of restriction endonucleases the presence of the corresponding methyltransferase is necessary to protect the cellular DNA from cleavage. For mutagenesis, sequencing, and overexpression of the *Eco*RI and *Eco*RV genes, the plasmids pRIF309+ (Wolfes et al., 1986) and pRVF03 (Thielking et al., 1991) or its derivative pRVF07 were used. pRVF07 was obtained by exchanging the upstream region of the *Eco*RV gene, including the Shine–Dalgarno sequence, by a sequence taken from the plasmid pRIF309+.

Both the *Eco*RV and the *Eco*RI genes are under control of the bacteriophage λp_L-promoter, and overexpression is induced by a temperature shift from 30 to 42 °C.

Mutagenesis. Site-directed mutagenesis was performed according to the gapped-duplex protocol (Kramer et al., 1984); for details, see Geiger et al. (1989), Alves et al. (1989a), and Thielking et al. (1991). Positive clones were screened by restriction enzyme analysis and verified by sequencing the entire gene.

Purification of EcoRV and EcoRI Proteins. Wild-type *Eco*RV and *Eco*RV mutants were isolated as described by Thielking et al. (1991). Wild-type *Eco*RV as well as the P73G, D90E, and K92E mutants were purified to homogeneity, while the preparations of the E45A, E45D, E45Q, P73A, D74A, D74E, D74N, D90A, D90N, D90T, K92A, and K92Q mutants were enriched in *Eco*RV to about 30%, and devoid of nonspecific nucleases. While the *Eco*RV concentration of homogeneous protein preparations was determined using the extinction coefficient of *Eco*RV (D'Arcy et al., 1985), the *Eco*RV content of the other preparations was determined by densitometric scanning of SDS–PAGE gels.

All experiments with *Eco*RI mutants were carried out with crude extracts of *E. coli* TGE900 cells producing the mutant proteins. TGE900 [pRIF309+, pECOR4] cells were grown in LB medium with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 30 °C to an optical density of 1 A_{600nm} (1 cm). The incubation was then continued for 2.5 h at 42 °C. Cells were harvested by centrifugation, washed with STE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.1 M NaCl), and resuspended in TAS buffer (10 mM Tris-acetate, pH 7.8, and 0.75 M sucrose). After addition of 100 μg/mL lysozyme, they were incubated for 10 min at room temperature. The suspension was centrifuged at 6000*g* for 30 min at 4 °C; 0.1 volume of buffer A [30 mM potassium phosphate, pH 7.2, 1 mM EDTA, 0.1 mM DTT, and 0.01% (v/v) Lubrol] containing 1 M NaCl was added to the supernatant. The solution was dialyzed overnight against buffer A containing 0.1 M NaCl and 70% (v/v) glycerol and stored at −20 °C. The concentration of the *Eco*RI mutants in these preparations was determined similarly as described above for *Eco*RV.

Circular Dichroism Spectroscopy. Circular dichroism spectra of wild-type *Eco*RV and the P73G, D90E, and K92E mutants were measured as described recently (Geiger et al., 1989).

Gel Shift Experiments. A 1–10 nM aliquot of 5'-^{32}P-labeled 377mer (the HPLC-purified *Eco*RI/*Bam*HI fragment of pAT153, kindly supplied by A. Landgraf) or 25 nM 5'-^{32}P-labeled 20mer d(CATTGTTAGATATCATACAC)·d(CGTGTATGATATCTAACAAT) was incubated with wild-type *Eco*RV or *Eco*RV mutants in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM spermine, and 0.1 mg/mL bovine serum albumin) for 15 min at 25 °C. To 25 μL of this mixture was added 5 μL of loading buffer [40% (w/v) sucrose, 0.25% (w/v) xylene cyanole, and 0.15% (w/v) azorubin in binding buffer]. Electrophoresis was carried out on 20 × 20 cm 6% polyacrylamide gels at room temperature with 50 mM Tris-borate, pH 8.0, and 1.25 mM EDTA, for 10 h at 100 V. Bands were visualized by autoradiography.

Similar experiments were carried out with wild-type *Eco*RI and *Eco*RI mutants using the 5′-^{32}P-labeled 16 mer d-(GTCCTGAATTCATTAG)·d(CTAATGAATTCAGGAC) which corresponds to one of the *Eco*RI sites in λ DNA.

DNA Cleavage Assays. For DNA cleavage assays, appropriate dilutions of wild-type *Eco*RV or *Eco*RV mutants were added to one of the following assay mixtures (final volume 15 μL): normal mix, 2 μg of pATRV DNA (a derivative of pAT153 with an additional *Eco*RV site), 20 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 10 mM $MgCl_2$; Mn^{2+} mix, 2 μg of pATRV DNA, 20 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 1 mM $MnCl_2$. Samples were incubated at 37 °C, aliquots were taken at various time intervals and analyzed by agarose gel electrophoresis. For quantification, the ethidium bromide stained gels were photographed and the negatives scanned using a laser densitometer. *Eco*RV activity is given in relative units defined by the rate of disappearance of supercoiled DNA upon incubation of *Eco*RV mutants with pATRV DNA at 37 °C, normalized by the rate determined with wild-type *Eco*RV.

Similar assays were carried out with wild-type *Eco*RI and *Eco*RI mutants using pTR54 DNA (a derivative of pMC9 which contains two *Eco*RI sites).

Results

The crystallographic analysis of the *Eco*RV–d(GGGATATCCC) complex (Winkler et al., 1992) has revealed the presence of a cluster of three acidic amino acid residues (Glu45, Asp74, and Asp90) and one basic amino acid residue (Lys92) in proximity to the scissile phosphodiester bond. In order to analyze the functional role of these residues, we have produced mutants at these positions. The acidic amino acid residues were substituted by their homologues as well as by the corresponding amides and by Ala, Asp90 also by Thr. Lys92 was substituted by Ala, Gln, and Glu. In addition, we have changed Pro73 to Gly and Ala, respectively. The mutant proteins were purified as indicated under Experimental Procedures and analyzed for their DNA binding and cleavage activity.

DNA Binding Experiments with EcoRV. All mutants with one exception (P73G) bind to DNA with detectable affinity ($K_{ass} > 10^5$ M^{-1}), as judged by gel retardation assays (Garner & Revzin, 1981; Fried & Crothers, 1981). Depending on the concentration of *Eco*RV proteins, more than one shifted band appears with a 377 bp DNA fragment. As this fragment contains only one canonical *Eco*RV site, binding must also occur at nonspecific sites. This phenomenon has already been described by Taylor et al. (1991) for wild-type *Eco*RV and been interpreted to be due to the fact that *Eco*RV binds all DNA sequences with equal affinity, the intrinsic affinity being approximately 10^6 M^{-1}. Figure 2A shows a representative gel shift experiment with wild-type *Eco*RV and the D90A mutant.

While all mutants except P73G bind to the 377 bp fragment under the conditions chosen, there are quantitative differences. It is particularly interesting that the K92E mutant binds DNA more weakly, whereas the D74A, D74N, D90A, and D90N mutants bind DNA more strongly than wild-type *Eco*RV. Different affinities are also seen with an oligodeoxynucleotide substrate (Figure 2B). These are to be expected for these mutants, because attractive interactions that might exist between Lys92 and the DNA are abolished in the K92E mutant, and possible repulsive interactions between Asp74 or Asp90 and the DNA are relieved in the D74A, D74N, D90A, and D90N mutants. D90A, for example, binds 2500-fold more strongly to specific DNA than wild-type *Eco*RV (Thielking et al., 1992). We attribute the impaired DNA binding of the

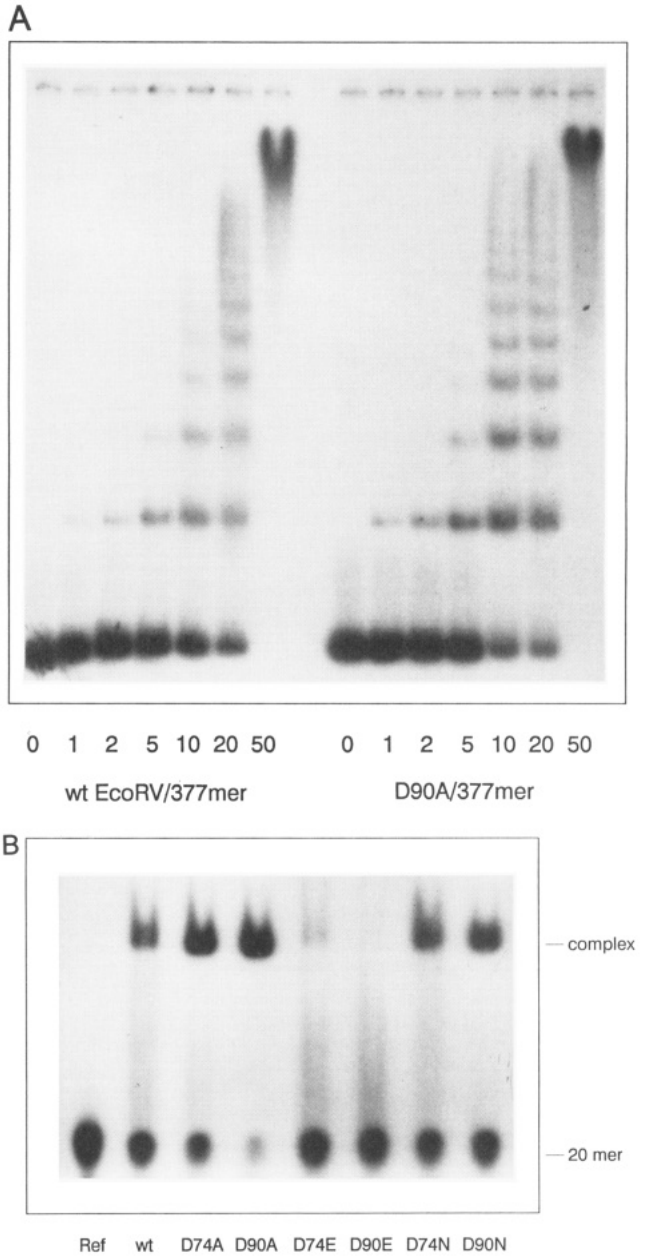

FIGURE 2: Gel retardation experiments with *Eco*RV–DNA complexes. In (A), gel retardation experiments are shown for wild-type *Eco*RV and the D90A mutant. 2.5 nM ^{32}P-labeled 377 bp fragment was incubated with 0-, 2.5-, 5-, 10-, 20-, and 50-fold molar excess of wild-type *Eco*RV (left) and the D90A mutant (right), respectively. In (B), gel retardation experiments are shown for wild-type *Eco*RV and the D74A, D90A, D74E, D90E, D74N, and D90N mutants. 25 nM ^{32}P-labeled 20mer was incubated with no (reference) or 125 nM aliquots of the mutants, respectively. Under these conditions, complex formation is not or barely detectable with the D74E and D90E mutants. Complex formation was analyzed on 6% polyacrylamide gels.

P73G mutant to be at least in part due to an altered protein structure. Circular dichroism spectroscopy indicates that P73G has a different secondary structure composition than the D90E or K92E mutants or wild-type *Eco*RV (Figure 3). Furthermore, the P73G mutant shows a different chromatographic behavior than wild-type *Eco*RV.

DNA Cleavage Experiments with EcoRV. The DNA cleavage activity of *Eco*RV and *Eco*RV mutants was determined with pATRV as substrate, in the presence of Mg^{2+} or Mn^{2+}, respectively. It must be emphasized that those mutants that show detectable activity under normal buffer conditions (cf. Experimental Procedures) are specific for their canonical recognition site, because in all cases the canonical *Eco*RV

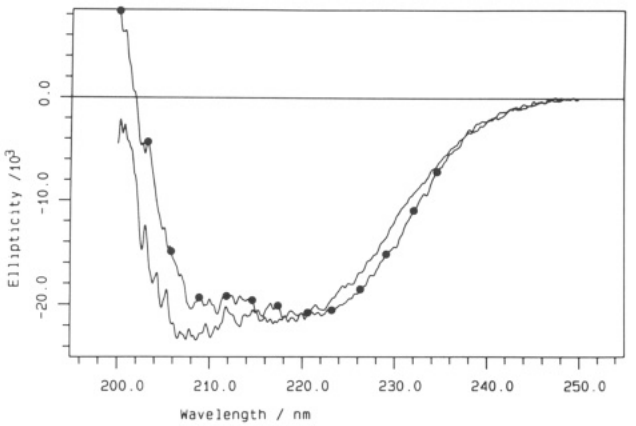

FIGURE 3: Circular dichroism spectra of the P73G and D90E mutants. The spectra were recorded with 0.145 mg/mL P73G mutant (●) and 0.169 mg/mL D90E mutant, respectively, in 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl at ambient temperature. The D90E spectrum is identical within the limits of error to the K92E and the wild-type *Eco*RV spectrum (Selent 1988).

cleavage pattern is obtained. Furthermore, all mutants except D74E and D90E have an increased proportion of nicking in comparison to wild-type *Eco*RV, which leads to the intermediate accumulation of the open circular form of pATRV.

(A) Negatively Charged Residues near the Scissile Bond. The strongest effects on DNA cleavage result from substitution of Asp74 and Asp90 by neutral amino acids. The exchange of Asp by Ala in these positions leads to mutants of undetectably low activity which is not due to an inability to bind DNA (described above) but rather reflects an interference with processes following binding. This could imply that at this position the carboxyl function or part of it is necessary for catalysis. This reasoning is in line with the finding that the exchange of Asp by Glu at these positions is not as deleterious as the exchange by Ala: D90E is as active as wild-type *Eco*RV; D74E shows substantial activity. The D74N, D90N, and D90T mutants, on the other hand, are inactive. These results make it clear that the carboxylate groups of Asp74 and Asp90 must be preserved for catalytic activity. Substitution of Glu45 by Ala or Gln strongly affects the activity of the enzyme, but not as dramatically as substitution of Asp74 or Asp90 by Ala or Asn. A tight fit at position Glu45 seems to be as important as the negative charge, because both the E45Q and E45D mutants are of similarly reduced activity. The results presented so far are compatible with a mechanism which assigns the function of activation of water to Asp74, Asp90, and possibly Glu45. Whether any of these amino acids serves directly as a proton acceptor or merely as a ligand for Mg^{2+}, which may be necessary to polarize the P–O bond and to bind the attacking water molecule, cannot be decided at present.

(B) Positively Charged Residue near the Scissile Bond. Mutations at position Lys92 are nearly as deleterious as at positions Asp74 and Asp90. The substitution of Lys by Gln, Ala, or Glu renders the enzyme inactive or nearly inactive. It is surprising that these mutations lead to similarly strong effects. If the positive charge of Lys92 were essential for *Eco*RV activity, one would expect the reversal of charge to produce a larger effect than its neutralization. This result can be understood in light of a set of DNA cleavage experiments carried out in the presence of Mn^{2+} instead of Mg^{2+}. Mn^{2+} can efficiently replace Mg^{2+} as a cofactor for the *Eco*RV-catalyzed cleavage of DNA, although in the presence of Mn^{2+} DNA cleavage with wild-type *Eco*RV is also observed at noncanonical sites ("star"-activity) (Halford et al., 1986).

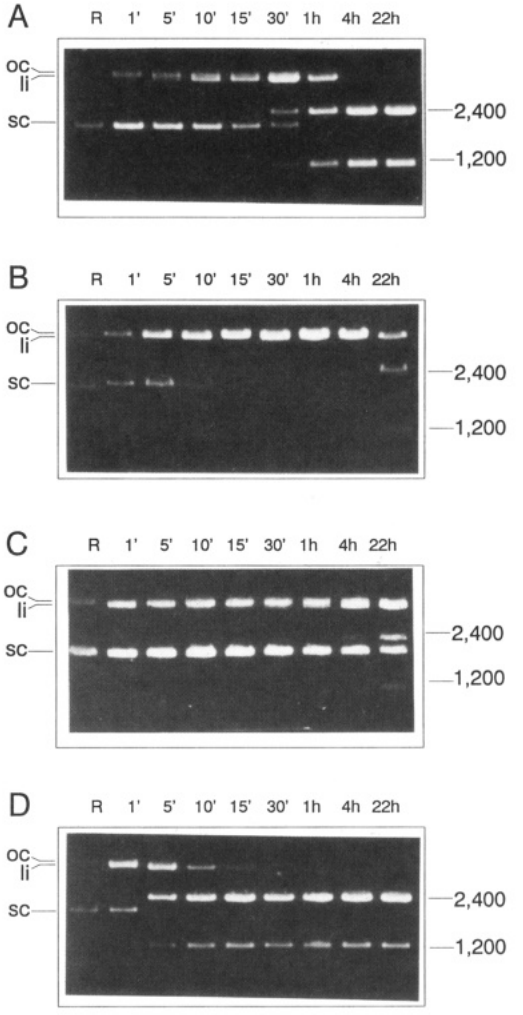

FIGURE 4: Time course of DNA cleavage by wild-type *Eco*RV and the K92E mutant under normal and "star" buffer conditions. (A) 165 nM pATRV DNA was incubated in normal mix (cf. Experimental Procedures) at 37 °C with 0.4 nM wild-type *Eco*RV (A) or 1200 nM K92E mutant (B). (B) 165 nM pATRV DNA was incubated in Mn^{2+} mix (cf. Experimental Procedures) at 37 °C with 0.4 nM wild-type *Eco*RV (C) and 120 nM K92E mutant (D). Under both buffer conditions, the substrate plasmid DNA [supercoiled (sc) and open circular (oc)], which contains two *Eco*RV sites, is cleaved to give an intermediate [linear (li)] and two products (2400 and 1200 bp, respectively).

Table I: Relative DNA Cleavage Activity of Active-Site Mutants of *Eco*RV

enzyme	relative activity	
	normal mix	Mn^{2+} mix
wild type	1	1/16
E45A	1/10000	1/10000
E45D	1/300	1/300
E45Q	1/300	1/800
P73A	1/1000	1/50
P73G	no cleavage	1/50
D74A	no cleavage	no cleavage
D74E	1/65	1/130
D74N	no cleavage	no cleavage
D90A	no cleavage	no cleavage
D90E	1	1
D90N	no cleavage	no cleavage
D90T	no cleavage	no cleavage
K92A	1/10000	1/10000
K92E	1/1500	1/25
K92Q	no cleavage	no cleavage

Mn^{2+} leads to a large acceleration of DNA cleavage by K92E as can be seen from Figure 4; this is not the case with K92Q and K92A (Table I). It seems that the function of Lys92 can

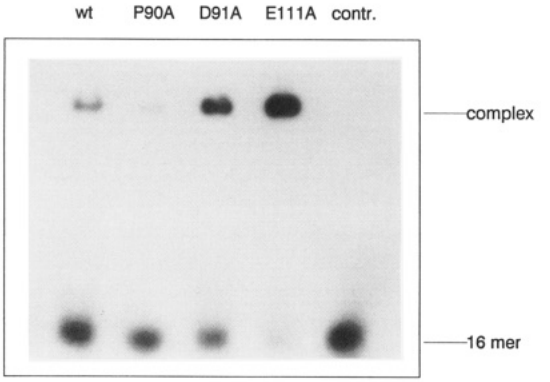

FIGURE 5: Gel retardation experiments with *Eco*RI–DNA complexes. 50 nM ^{32}P-labeled 16mer was incubated with an equimolar concentration of wild-type *Eco*RI and the P90A, D91A, and E111A mutants or, as a control, with a TGE900 extract not producing *Eco*RI, respectively. Complex formation was analyzed on 12% polyacrylamide gels.

be fulfilled by Glu92/Mn^{2+}. These results suggest a role for Lys92, i.e., compensating the extra negative charge of the pentacovalent phosphorus during the transition state. This role can be taken over by a firmly bound Mn^{2+} ion with the K92E mutant. It must be emphasized that there are no other basic amino acid residues sufficiently close to the DNA to fulfill this function.

(*C*) *Proline Residue near the Scissile Bond.* The *Eco*RV residues Asp74, Asp90, and Lys92 are part of a sequence motif (Pro-Asp...Asp/Glu-X-Lys) present in many restriction endonucleases, including *Eco*RI and its isoschizomer *Rsr*I (Thielking et al., 1991). We have, therefore, also analyzed the effect of replacements of Pro73 in *Eco*RV, and started a mutational analysis of this region in *Eco*RI. As shown in Table I, the P73A mutant of *Eco*RV shows a considerable decrease in activity. The P73G mutant is inactive. The inability of P73G to cleave DNA is presumably due to an impaired DNA binding (described above). The low activity of the P73A mutant, which binds to DNA, suggests that Pro73 has an important structural role in positioning its neighbor Asp74 in the catalytic center of *Eco*RV.

As can be seen in Table I, the P73A and P73G mutants are also activated by Mn^{2+}. This is particularly interesting for the P73G mutant which has an altered secondary structure, and shows no detectable DNA binding activity in the absence of Mg^{2+} and no DNA cleavage activity in the presence of Mg^{2+}. We assume that with these mutants, Mn^{2+} *and* DNA shift an equilibrium between different conformations by binding with preference to the catalytically competent conformer.

DNA Binding and Cleavage Experiments with EcoRI. The importance of the Pro-Asp...Asp/Glu-X-Lys motif for the catalytic activity of *Eco*RI was analyzed by DNA cleavage experiments with extracts of *E. coli* cells producing the mutants P90A, D91A, D91N, E111A, and E111K. Substitution of Pro90 by Ala leads to an approximately 10–100-fold decrease of DNA cleavage activity. The finding that the Pro to Ala exchange is at least 10 times more deleterious in *Eco*RV than in *Eco*RI might be due to the fact that Pro73 in *Eco*RV is cis while Pro90 in *Eco*RI has been assigned to be trans at the present level of resolution. All other mutants are by at least 2–3 orders of magnitude less active than wild-type *Eco*RI (data not shown). The low activity is not due to impaired DNA binding as shown by gel retardation assays (Figure 5). The D91A mutant and in particular the E111A mutant show a higher affinity for DNA than wild-type *Eco*RI. In addition, *Eco*RI mutants at position Glu111 have been described previously and shown to be by 2–5 orders of magnitude less active than wild-type *Eco*RI, depending on the mutant and the assay conditions (Wolfes et al., 1986; King et al., 1989; Wright et al., 1989). Similarly as observed here for the E111A mutant, the E111Q mutant binds more firmly to its DNA substrate than wild-type *Eco*RI (Wright et al., 1989), presumably because repulsive interactions between the DNA backbone and the Glu111 residue of *Eco*RI are relieved by the mutation. These data support the suggestion that Asp91 and Glu111 in *Eco*RI have a similar function as Asp74 and Asp90 in *Eco*RV. We have not yet obtained mutants at position Lys113 in *Eco*RI, the presumptive equivalent to Lys92 in *Eco*RV.

Discussion

In the study presented here, the question has been addressed which amino acids in *Eco*RV participate in phosphodiester bond cleavage. The starting point of our investigation was the crystal structure of an *Eco*RV–DNA complex (Winkler et al., 1992) which led to the identification of those amino acid residues located sufficiently close to the scissile phosphodiester bond to be candidates for active-site residues. Because of the structural homology of this region in *Eco*RV and *Eco*RI (Kim et al., 1990), the focus of our attention was directed to those residues in *Eco*RV that have counterparts in *Eco*RI. These *Eco*RV residues were mutated, and the effects of the mutations on DNA binding and cleavage were analyzed. To demonstrate that the structural homology of the presumptive catalytic sites of *Eco*RV and *Eco*RI is of functional relevance, we have included in our study also a preliminary analysis of some *Eco*RI mutants. On the basis of the results of this study and previous mechanistic work from several laboratories, it should be possible to propose a mechanism for DNA cleavage by *Eco*RV and *Eco*RI. For this purpose, a comparison with other nucleases may be helpful.

Proposals for the mechanism of phosphodiester bond hydrolysis by several nucleases (RNases and DNases, exo- and endonucleases) have been put forward [see review by Saenger (1991)]. Some of these nucleases may serve as models for *Eco*RV and *Eco*RI, because they need divalent metal ions for catalysis and have similar amino acid residues in their presumptive active sites as shown by their X-ray structure analyses:

(I) The 3′–5′ exonuclease activity of *E. coli* DNA polymerase I depends on two metal ions (Zn^{2+}, Mg^{2+}?). Four acidic amino acid residues are involved in metal ion binding and water activation (Beese & Steitz, 1991). Their substitution leads to almost inactive enzymes (Derbyshire et al., 1991). Stabilization of the pentacovalent phosphorus is thought to be mediated by one of the metal ions.

(II) The *Staphylococcus* nuclease is a Ca^{2+}-activated nuclease. Three acidic amino acid residues are ligands of the metal ion. One of them has been proposed to function as a general base by activating a Ca^{2+}-bound water molecule. A basic amino acid residue contributes to transition-state stabilization and serves as a general acid (Cotton et al., 1979; Serpersu et al., 1987, 1989; Hibler, 1987; Weber et al., 1991).

(III) The *Penicillium citrinum* P1 nuclease contains three Zn^{2+} ions, one of which is associated with the phosphate group and is coordinated by an Asp residue. An Arg residue is presumably involved in stabilization of the pentacovalent phosphorus (Volbeda et al., 1991).

(IV) The *E. coli* RNase H requires divalent cations, usually Mg^{2+}, for activity and contains three acidic amino acid residues which are essential for catalysis (Kanaya et al., 1990) and are conserved in other RNase H sequences. On the basis of these data and structural information, it is assumed that a similar

mechanism applies for RNase H as for the 3′–5′ exonuclease of DNA polymerase I (Yang et al., 1991).

We propose that *Eco*RV and *Eco*RI basically follow the same mechanism in cleaving nucleic acids as these four enzymes: a hydroxide ion, activated by a metal cation and an acidic amino acid residue, attacks to form a pentacovalent phosphorus intermediate stabilized by a cation (metal ion or basic amino acid residue); the P–O bond is cleaved with inversion of configuration (DNA polymerase I exonuclease, Gupta & Benkovic, 1984; nuclease P1, Potter et al., 1983a,b; *Eco*RI, Connolly et al., 1984; *Eco*RV, Grasby & Connolly, 1992). Two acidic amino acid residues (*Eco*RV, Asp74 and Asp90; *Eco*RI, Asp91 and Glu111), located in close proximity to the scissile phosphodiester bond, have been shown by site-directed mutagenesis to be indispensable for the catalytic function of *Eco*RV and *Eco*RI. With reference to the 3′–5′ exonuclease domain of *E. coli* DNA polymerase I, staphylococcal nuclease, nuclease P1, and RNase H, it seems reasonable to assume that these acidic amino acid residues are responsible for binding the metal ion cofactor which in turn polarizes the phosphorus to allow nucleophilic attack by a hydroxide ion. The evidence at this point is insufficient to decide which amino acid residue is responsible for generating the hydroxide ion. A third acidic amino acid, also not too far away from the scissile phosphodiester bond (*Eco*RV, Glu45; *Eco*RI, Glu144), is essential for the catalytic activity of these two restriction enzymes, not, however, to the same extent as the other two. In *Eco*RI, Glu144 is the only acidic amino acid except Glu111 and Asp91 within approximately 10-Å distance (C_α–P) from the scissile phosphodiester bond (Figure 1). On the basis of the revised *Eco*RI crystal structure, it has been proposed that Glu144 does not contact DNA directly but rather positions other residues that do so, such as Asn141, Arg145, Arg203, and Lys148 (Rosenberg, 1991). Whether this is also the case in the presence of Mg^{2+} is not clear at present. *Eco*RI mutants at this position have been described previously. The E144Q mutant is by 2–3 orders of magnitude less active than wild-type *Eco*RI (Wolfes et al., 1986; Hager et al., 1990). The E144D mutant, on the other hand, shows considerable enzymatic activity (Hager et al., 1990; Heitman & Model, 1990a). Other mutants have been characterized in vivo by Heitman and Model (1990a); their results support the conclusion that Glu144 plays an important role in catalysis by *Eco*RI. Whether Glu45 in *Eco*RV and Glu144 in *Eco*RI fulfill the same function in DNA cleavage by these enzymes remains to be seen.

A basic amino acid residue (Lys92) has also been identified in *Eco*RV to be essential for catalysis. As an *Eco*RV mutant in which Lys92 is substituted by Glu is active in the presence of Mn^{2+}, the function of Lys92 could be taken over by a Mn^{2+} ion bound to Glu92. Accordingly, this mutant may follow a two-metal-ion mechanism as proposed for the 3′–5′ exonuclease activity of the *E. coli* DNA polymerase I (Beese & Steitz, 1991). A change of mechanism due to a single amino acid replacement has already been observed with other enzymes. For example, in RNase T_1, His40 acts as a base catalyst when the true catalytic base Glu58 is replaced by Ala (Steyaert et al., 1990). In *Eco*RI, Lys113 may have a similar role as Lys92 in *Eco*RV, as suggested by a comparison of the two structures. By analogy with staphylococcal nuclease and nuclease P1, this residue might serve to stabilize the transition state in which a pentacovalent phosphorus is formed and/or to function as general acid. Our data with *Eco*RV support this notion. With *Eco*RI, we have not yet obtained mutants at position 113. A mutational analysis had been carried out previously, however, for the amino acid next to Lys113; His114, when replaced by Asn, leads to a 160-fold decrease in DNA cleavage activity, mainly due to a diminished k_{cat}, but with no decrease in specificity (R. Geiger, J. Alves, and A. Pingoud, unpublished results). If Lys113 is needed to stabilize the transition state, as we assume, amino acid substitutions in its vicinity are likely to affect the catalytic efficiency of *Eco*RI. The more drastic substitution of His114 by Tyr results in a mutant enzyme with promiscuous substrate specificity (Heitman & Model, 1990b). As His114 does not contact the phosphodiester backbone but points away from the DNA, these effects must be indirect ones. The peptide backbone of *Eco*RI at position 114 is within hydrogen-bonding distance of the phosphodiester bond between the two adenine residues of the recognition sequence; amino acid substitutions at position 114, hence, are likely to affect catalytic specificity. In addition, His114 is located next to Gln115 which is involved in a base-specific contact (Rosenberg, 1991), which may be disturbed when His114 is changed to Tyr.

Having identified amino acid residues involved in catalysis, it must be enquired how recognition is coupled with catalysis, or how the cleavage center of *Eco*RV is activated by substrate binding. We have recently established (Thielking et al., 1991) that amino acid residues located in two loop regions around positions 70 and 185, respectively, are responsible for DNA recognition. Some of them, like Asn188, are very close to the scissile phosphodiester bond; therefore, it is likely that they are involved both in DNA binding and in catalysis. Others are further away and, therefore, can only indirectly stimulate the active site. A possible trigger could be provided by Mg^{2+} binding: only when specific contacts between *Eco*RV and the DNA have been made during the recognition process, Asp74, Asp90, and the substrate are in a favorable orientation to bind Mg^{2+}. This model would explain why the substitution of Mn^{2+} for Mg^{2+} leads to DNA cleavage at degenerate sites ("star" sites): having a higher affinity for carboxylate ligands than Mg^{2+} (Martell & Smith, 1977) and having a slightly larger ionic radius, Mn^{2+} can even be bound under conditions when the metal ion binding site of *Eco*RV is in a suboptimal conformation, as it might be when *Eco*RV is interacting with an *Eco*RV "star" site. Supporting evidence for this conjecture comes from the recent work of Vermote and Halford (1992), who have shown that *Eco*RV has a higher affinity for Mn^{2+} than for Mg^{2+} when bound to a "star" site.

Because Mn^{2+} leads to a relaxed specificity also of *Eco*RI (Hsu & Berg, 1978), a similar explanation can be given for the activation of the catalytic center of *Eco*RI by its substrate DNA. The allosteric activation model proposed by McClarin et al. (1986), Terry et al. (1987), and Heitman and Model (1990b) suggests that the catalytic center of *Eco*RI is inactive in the nonspecific complex and becomes activated only upon substrate binding. As discussed by Rosenberg (1991), the *Eco*RI–DNA recognition complex is characterized by an extensive net working between the DNA and amino acid residues involved in recognition and catalysis. The proximity between residues involved in DNA recognition (e.g., Gln115 which contacts the methyl group of the inner thymidine residue) and Mg^{2+} binding (e.g., Glu111) supports the hypothesis of a coupling between recognition and cleavage via formation of a Mg^{2+} binding site. Coupling implies that following DNA binding a reorientation of the protein/DNA interface occurs which brings amino acid residues of the catalytic center in close proximity to the scissile phosphodiester bond. It can be assumed that this reorientation is optimal for canonical sites and suboptimal for degenerate sites, because Lesser et al. (1990)

have shown that the "footprint" of *Eco*RI on DNA is different depending on whether a canonical or a degenerate site is encountered.

Both for *Eco*RI and *Eco*RV, we assume that Mg^{2+} is only bound tightly when the DNA binding site is occupied, as it was shown similarly for the 3′–5′ exonuclease domain of polymerase I which binds Mn^{2+} 100-fold more tightly in the presence of TMP or dTMP (Mullen et al., 1990). Circumstantial evidence for this assumption has been provided by Halford and Goodall (1988) and Taylor and Halford (1989) for *Eco*RV. They have shown that the activity of *Eco*RV is governed by its affinity to Mg^{2+} and even more importantly that this enzyme has a lower affinity for Mg^{2+} when it is bound to a noncognate site. For *Eco*RI, Halford and Johnson (1983) and Alves et al. (1989b) have demonstrated in single-turnover experiments that cleavage proceeds faster with a preformed *Eco*RI–DNA complex to which Mg^{2+} is added than with *Eco*RI and Mg^{2+} to which DNA is added. It was argued that the preformed enzyme–DNA complex has already undergone some of the conformational transitions necessary for catalysis. On the basis of the previous discussion, we suggest that this conformational transition is responsible for formation of a strong Mg^{2+} binding site.

While it can be concluded from the results presented here that *Eco*RV and *Eco*RI basically follow the same mechanism in cleaving DNA, it should be emphasized that Mg^{2+} plays a more direct role in recognition for *Eco*RV than for *Eco*RI. In the absence of Mg^{2+}, *Eco*RV binds all DNA sequences with equal and moderate affinity (Taylor et al., 1991), while *Eco*RI shows a highly preferential strong binding to its recognition sequence (Halford & Johnson, 1980; Clore et al., 1982; Terry et al., 1983, 1985; Thielking et al., 1990; Lesser et al., 1990). In the presence of Mg^{2+}, on the other hand, *Eco*RV (Thielking et al., 1992), like *Eco*RI (Alves et al., 1989b), binds strongly to its recognition sequence. In conclusion, it is proposed for *Eco*RV and *Eco*RI that in the recognition complex Mg^{2+} is coordinated by two acidic amino acid residues, one of the phosphate oxygens, and possibly a water molecule. During catalysis, this or another water molecule is activated for an in-line attack on phosphorus which is supported by the stabilization of the developing extra negative charge by a basic amino acid residue nearby. This mechanism may be valid also for other restriction endonucleases.

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Identification of Critical Lysyl Residues in the Pyrophosphate-Dependent Phosphofructo-1-kinase of *Propionibacterium freudenreichii*†

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ABSTRACT: Pyrophosphate-dependent 6-phosphofructo-1-kinase (PP_i-PFK) from *Propionibacterium freudenreichii* was inactivated by low concentrations of the lysine-specific reagent pyridoxal phosphate (PLP) after sodium borohydride reduction. The substrates fructose 6-phosphate and fructose 1,6-bisphosphate protected against inactivation whereas inorganic pyrophosphate had little effect. An HPLC profile of a tryptic digest of PP_i-PFK modified at low concentrations of PLP showed a single major peak with only a small number of minor peaks. The major peak peptide was isolated and sequenced to obtain IGAGXTMVQK, where X represents a modified lysine residue, corresponding to Lys-315. Lys-315 was protected from reaction with PLP by fructose 1,6-bisphosphate. As indicated by HPLC maps of PP_i-PFK modified with varying concentrations of PLP, a direct correlation was observed between activity loss and the modification of Lys-315. Two of the minor peptide peaks were shown to contain Lys-80 and Lys-85, which were modified in a mutually exclusive manner. Partial protection against modification of these two residues was provided by $MgPP_i$. The data were used to adjust the sequence alignment of the *Propionibacterium* enzyme with that of ATP-dependent PFK of *Escherichia coli* to identify homologous residues in the substrate binding site. It is suggested that Lys-315 interacts with the 6-phosphate of fructose 6-phosphate and that Lys-80 and -85 may be located near the pyrophosphate binding site.

Recently, this laboratory described the amino acid sequence of the pyrophosphate-dependent phosphofructokinase (PP_i-PFK)[1] from *Propionibacterium freudenreichii* (Ladror et al., 1991). The properties of this enzyme differ substantially from the major family of ATP-dependent phosphofructokinases in that it is dimeric as opposed to being a tetramer and that it displays no allosteric properties. On the other hand, a low but significant level of sequence identity with *Escherichia coli* PFK was established to indicate that the PP_i-dependent and the ATP-dependent enzymes are homologous (Ladror et al., 1991). While the overall identity was only 23%, the alignment of the amino-terminal half of PP_i-PFK showed 30% identity, with a much lower similarity in the carboxyl-terminal half. In addition, seven residues which have been shown to be involved in the binding of the sugar phosphate substrate by the ATP-dependent *E. coli* enzyme (Shirakihara & Evans, 1988) could be readily aligned with identical residues in the amino-terminal half of the PP_i-dependent enzyme. Three basic residues found

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[1] Abbreviations: PFK, 6-phosphofructo-1-kinase; Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; DTT, dithiothreitol; PP_i, inorganic pyrophosphate; PLP, pyridoxal phosphate.

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