

Hypothesis

On the catalytic mechanism of *EcoRI* and *EcoRV*

A detailed proposal based on biochemical results, structural data and molecular modelling*

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Received 6 April 1992

EcoRI and *EcoRV* have a very similar active site, as is apparent from a comparison of the structures of their respective protein–DNA complexes. Based on structural and mechanistic data, as well as detailed molecular modelling presented here, a mechanism for the DNA cleavage by these enzymes is suggested in which the attacking water molecule is activated by the phosphate group 3' to the scissile phosphodiester bond, and in which the leaving group is protonated by a water molecule associated with the essential cofactor, Mg^{2+} . The mechanism proposed may also apply to other nucleases.

EcoRI; *EcoRV*; DNA cleavage; Catalytic mechanism; Molecular modelling

1. INTRODUCTION

X-ray crystallography has contributed tremendously to the understanding of protein nucleic acid interactions, particularly for DNA binding proteins (for review cf. [1–3]). Enzymes acting on DNA, in addition to being sequence-specific binding proteins, fulfill a catalytic function. Understanding the details of their mechanism of action requires structural information. Yet even if an X-ray structure analysis of an enzyme substrate complex is available the deduction of a mechanism may not be straightforward because, although such structures often are good models for the ground state of enzyme–substrate complexes, they contribute less to the understanding of the transition state. Examples are the restriction enzymes, *EcoRI* and *EcoRV*, which, in the presence of Mg^{2+} , catalyze the specific hydrolysis of phosphodiester bonds in double-stranded DNA at the palindromic sites G/AATTC [4,5] and GAT/ATC [6,7], respectively. The X-ray structure analysis of co-crystals of these enzymes in complex with their respective cognate oligodeoxynucleotide substrates are available [8,9]. As the crystals were grown in the absence of the essen-

tial cofactor, Mg^{2+} , to prevent DNA cleavage they presumably do not represent the ground state of the complex before catalysis. Whereas the *EcoRI*–DNA co-crystal structure, however, may be regarded as the structure of a recognition complex (because *EcoRI* binds specifically to DNA in the absence of Mg^{2+}) this does not equally hold for *EcoRV*, which in the absence of Mg^{2+} does not specifically bind DNA [10,11]. This means that considerable differences must exist between the structures of the *EcoRI*- and, in particular, *EcoRV*–DNA complexes, as seen in the X-ray structure analyses and the structures of the catalytically competent enzyme–substrate complexes. In the present contribution an analysis is presented which utilizes structural and mechanistic information to deduce likely structures for the active complexes and to present a mechanism for DNA cleavage by *EcoRI* and *EcoRV*.

2. RESULTS AND DISCUSSION

Site-directed mutagenesis studies have shown that the carboxyl-group of Glu¹¹¹ is essential for the catalytic action of *EcoRI* [12–14]. Later a structural homology between *EcoRI* and *EcoRV* in the vicinity of the phosphate group to be cleaved was noticed, consisting of four amino acids located in a similar steric arrangement (Pro⁹⁰, Asp⁹¹, Glu¹¹¹ and Lys¹¹³ in *EcoRI* and Pro⁷³, Asp⁷⁴, Asp⁹⁰ and Lys⁹² in *EcoRV*, Fig. 1) [15]. Subsequently it was demonstrated by a mutational analysis

*This paper is dedicated to Manfred Eigen on the occasion of his 65th birthday.

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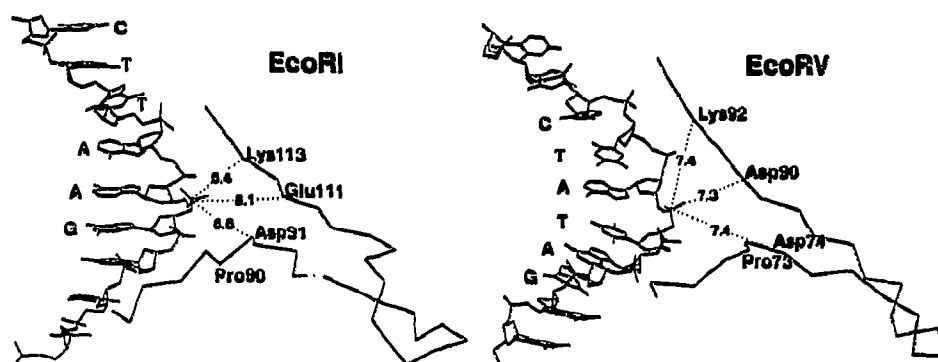


Fig. 1. Homologous amino acids in the presumptive active sites of *EcoRI* and *EcoRV*. The figure depicts part of the structure of *EcoRI* (left) and *EcoRV* (right) using the coordinates deposited in the Brookhaven protein databank. For clarity, only one strand of the DNA is shown. The polypeptide backbone is represented by the $C\alpha$ positions. Indicated are the $C\alpha$ -P distances between several residues of *EcoRI* and *EcoRV*, respectively, and the scissile phosphodiester bond.

that the two acidic amino acids of this homologous region in *EcoRV* and *EcoRI* are important for catalysis, as well as Lys⁹² in *EcoRV* (Lys¹¹³ has not yet been analyzed in *EcoRI*) [16]. It was concluded, therefore, that the general catalytic mechanism of both enzymes is similar, although they differ in their recognition sequence and the position of cleavage within this sequence.

The similarity between the mechanism of DNA cleavage by *EcoRI* and *EcoRV* is also apparent in the stereochemical course of the reaction. Both enzymes hydrolyse the phosphodiester bond on its 3'-side leaving a 5'-phosphate. Cleavage occurs with inversion of configuration at the phosphorous atom [17,18]. This finding is compatible with a direct S_N2 attack of an activated water in line with the 3'-OH leaving group (Fig. 2). This mechanism requires:

- (i) a Lewis acid that polarizes the non-bridging P-O bonds, thereby increasing the electrophilicity of the phosphorus atom;
- (ii) a general base that activates the attacking water molecule by increasing the negative partial

charge of the oxygen atom and strengthens its nucleophilicity;

- (iii) a Lewis acid that stabilizes the extra negative charge of the pentacoordinated phosphorous during the transition state;
- (iv) a Brönsted acid that protonates the leaving group to decrease its nucleophilicity.

Probably, Mg^{2+} fulfills the role of the Lewis acid (see i) because soaking of Mg^{2+} or Mn^{2+} into *EcoRI*-DNA co-crystals leads to in situ cleavage of the DNA and leaves the divalent cation between Glu¹¹¹ and the post-reactive phosphate [19]. A similar experiment carried out with *EcoRV* does not result in DNA cleavage in situ, but leads to the binding of the metal ion near the scissile phosphodiester bond (F. Winkler, pers. commun.). An oxygen atom of the phosphate group can be part of the coordination sphere of the Mg^{2+} , the cation thereby effectively compensates for the negative charge of the phosphate group. This implies that the Mg^{2+} binding site is formed by the enzyme and the DNA similarly, as proposed for the exonuclease domain of the Klenow polymerase [20-23]. Asp⁹¹ and Glu¹¹¹ in *EcoRI*

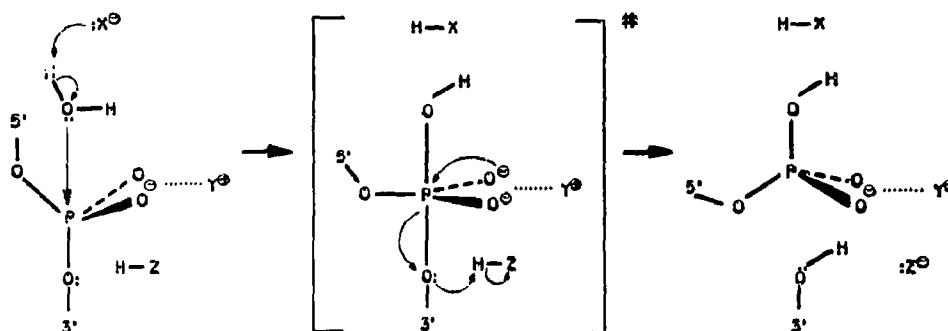


Fig. 2. A general mechanism for DNA cleavage by *EcoRI* and *EcoRV*. An activated water molecule attacks the phosphorous atom in a S_N2 reaction which proceeds with inversion of configuration, as indicated. The general acid-base catalysis by X^- and $H-Z$ is hypothetical. There is good evidence for Y^+ to be Mg^{2+} . The extra negative charge of the penta-coordinated phosphorous in the transition state presumably is neutralized by a basic amino acid residue (Lys¹¹³ in *EcoRI*, Lys⁹² in *EcoRV*; not shown here).

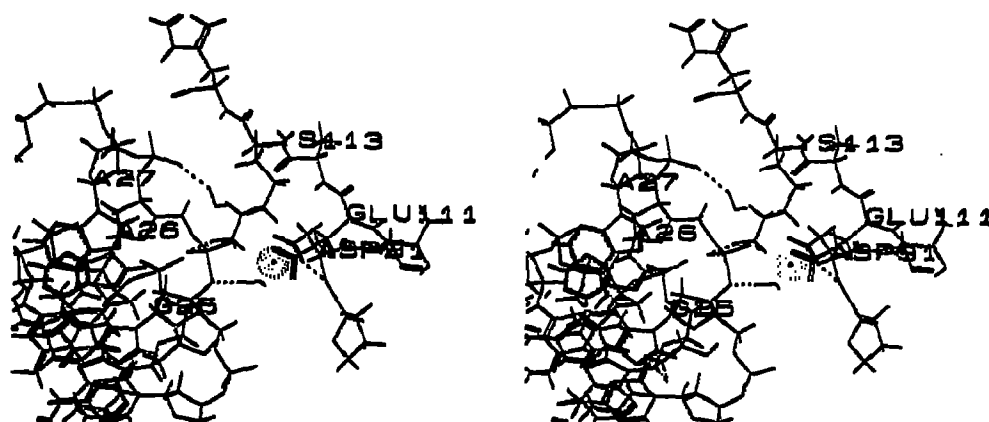


Fig. 3. Stereo drawing of a molecular model of the active site of *EcoRI*. G25, A26 and A27 are part of the recognition sequence G/AATTC of *EcoRI*. The Mg^{2+} ion is coordinated by Asp⁹², Glu¹¹¹, one non-bridging oxygen atom of the phosphate group between G25 and A26 and one water molecule which is additionally hydrogen-bonded to the bridging G25-3'-oxygen atom. The attacking water is hydrogen-bonded to a non-bridging oxygen atom of the phosphate group between A26 and A27.

and Asp⁷⁴ and Asp⁹⁰ in *EcoRV* are involved in Mg^{2+} binding, as shown by site-directed mutagenesis experiments [11,16]. Lys¹¹³ (*EcoRI*) and Lys⁹² (*EcoRV*), respectively, can act as the Lewis acid (see iii) and stabilize the transition state in addition to assisting the Mg^{2+} ion in positioning the phosphate and making the phosphorous more electrophilic.

Unfortunately, neither the *EcoRI*-DNA nor the *EcoRV*-DNA cocrystal structures have good candidates in the surrounding region of the scissile bond for the two other required catalytic functions, namely the general base that activates the attacking water (see ii), and the Brønsted acid that protonates the leaving group (see iv) [9,19]. In the case of *EcoRV* this is not surprising because structural changes of the complex are likely to occur upon Mg^{2+} binding, since specific association of the enzyme to DNA requires Mg^{2+} and DNA cleavage does not occur in crystals after Mg^{2+} is diffused into them. In the case of *EcoRI*, however, cleavage occurs in the crystals after soaking in Mg^{2+} ; structural changes in the course of the catalysis, therefore, should be smaller than with *EcoRV*, and the two missing functional groups are expected to be near the reactive phosphate.

In order to identify likely candidates for the general base (ii) and the Brønsted acid (iv), the structures of the active center of *EcoRI* and *EcoRV* in the presence of Mg^{2+} and water were simulated by molecular modelling. The simulation was done using an ESV (Evans & Sutherland, St. Louis) work station equipped with the molecular modelling software SYBYL 5.4 (TRIPOS, St. Louis) containing energy minimizer and molecular dynamics modules employing the TRIPOS force field [24]. Starting with the published coordinates of the *EcoRI*(C α -atoms)-DNA complex (Brookhaven data bank entry 1RIE) we added the side chains of the protein using all available information on protein-DNA and protein-protein interactions [19] in several rounds

of energy minimization. The structure obtained accommodates all these contacts and is free of unfavourable van der Waals contacts and angle- or torsion stress. In the next step we added one Mg^{2+} ion in the center of a triangle formed by the phosphorus atom to be attacked, and the carbon atoms of the carboxyl groups of Asp⁹¹ and Glu¹¹¹ that most likely form the Mg^{2+} binding site. We also added one water molecule at a position such that it could attack the phosphate group in line with the 3'-OH leaving group. Energy-minimized structures showed that neither the attacking water molecule nor the second non-bridging oxygen atom of the phosphate group, or amino acids other than Asp⁹¹ or Glu¹¹¹, are part of the inner hydration sphere of the Mg^{2+} . Therefore, a second water molecule was included in the molecular modelling to allow for a hexagonal hydration sphere of Mg^{2+} . Several rounds of energy minimization and molecular dynamic runs yielded a low energy structure (Fig. 3) that allows the suggestion of candidates for the general base (ii) and the Brønsted acid (iv) involved in the *EcoRI* catalyzed DNA cleavage.

A similar molecular modelling was carried out for *EcoRV* starting with the structure of the specific *EcoRV*-DNA complex (Brookhaven data bank entry 3RVE). Since this data set contains the side chain coordinates, only a Mg^{2+} ion and two water molecules had to be modelled into the complex. Not unexpectedly local rearrangements were necessary to generate a suitable Mg^{2+} binding site. Following minimization a low energy structure was obtained (Fig. 4).

In principle, the structural arrangement seen for *EcoRI* and *EcoRV* is very similar. The attacking water can form a hydrogen bond to one oxygen atom of the phosphate group, which is located on the 3'-side of the scissile phosphodiester bond. This phosphate can act as a base and abstract a proton from the attacking water. Interestingly, in the crystal structures of *EcoRI* and *EcoRV*, this phosphate is not directly contacted by the

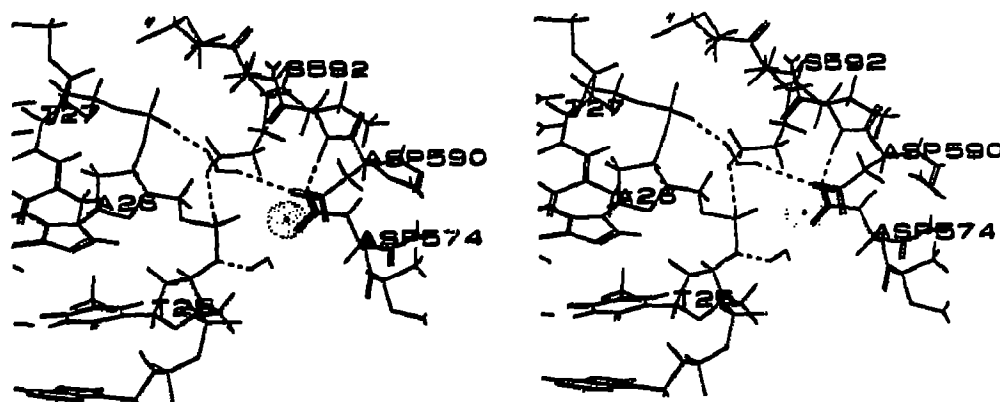


Fig. 4. Stereo drawing of a molecular model of the active site of *EcoRV*. T25, A26 and T27 are part of the recognition sequence GAT/ATC of *EcoRV*; Asp⁵⁷⁴, Asp⁵⁹⁰ and Lys⁵⁹² represent Asp⁷⁴, Asp⁹⁰ and Lys⁹², respectively. The Mg²⁺ ion is coordinated by Asp⁷⁴, Asp⁹⁰, one non-bridging oxygen atom of the phosphate group between T25 and A26 and one water molecule which is additionally hydrogen-bonded to the bridging T25-3'-oxygen atom. The attacking water is hydrogen-bonded to a non-bridging oxygen atom of the phosphate group between A26 and T27.

enzyme, a result which in the case of *EcoRI* was confirmed by ethylation interference experiments [25]. This finding suggests that this phosphate group is available to form the proposed hydrogen bond. Although the pK_i of D-ribose-5-dihydrogen phosphate is 2 [26], it might well be that the environment of the protein DNA interface results in a pK shift which would render this phosphate a reasonable proton acceptor at neutral pH and make it suitable for the role to activate water. Fig. 3 and Fig. 4 show that the second water molecule, which is part of the inner hydration sphere of the Mg²⁺ ion, can form a strong hydrogen bond to the 3'-OH group. Since it is possible that the pK of a [Mg²⁺ aquo]-complex in proteins is significantly lower than in water (pK = 10.2 [27]), it seems possible that this Mg²⁺-bound water protonates the leaving group through a favourable six-membered cycle.

The assignment of the neighbouring phosphate as responsible for water activation easily explains how specific DNA binding activates the enzyme to catalyze DNA cleavage. Recognition leads to considerable distortion of the DNA substrate [8,9], brings the water-activating phosphate in an optimum position for catalysis and thereby couples recognition and catalysis. Nevertheless there are alternatives which have to be discussed. While Asp⁹¹ in *EcoRI* and Asp⁷⁴ in *EcoRV* are too far away from the attacking water it may be possible that the other acidic amino acid residues (Glu¹¹¹ and Asp⁹⁰, respectively) activate the water molecule similarly, as suggested for other nucleases (RNaseH: [28], 3'-5' nuclease domain of Klenow polymerase: [22]). It must be emphasized that all candidates for water activation require a higher basicity of the proposed proton acceptors than that observed in the isolated groups dissolved in water. For *EcoRI* and *EcoRV*, the pK shift seems to be more reasonable for the phosphate group at the protein DNA interface where it is not contacted by cationic groups, than for a carboxyl group that

forms an ion pair with a Mg²⁺. In *EcoRV* Asn¹⁸⁸ has been suggested to position the attacking nucleophile [9]. It seems that this asparagine is not sufficiently basic to function also as a water-activating base. Additionally the *EcoRV* mutant, N188A, retains a reduced activity which makes it very unlikely that Asn¹⁸⁸ has this role in the catalytic mechanism [15,29].

We are planning to employ the diastereomers of phosphorothioate and methyl phosphonate containing oligodeoxynucleotides as substrates to investigate whether water activation in *EcoRI* and *EcoRV* is achieved by interaction of the water molecule with one oxygen atom of the adjacent phosphate group. Yet even at this state of model building, where experimental test systems are not presently available, we would like to draw attention towards the possibility that, in *EcoRI*, *EcoRV* or perhaps in other nucleases as well, the water activating base may be a neighbouring phosphate group of the DNA substrate and not an amino acid residue of the enzyme. Ribozymes, which also require Mg²⁺ as an essential cofactor, demonstrate that components of nucleic acids can function in such a manner [30,31].

Acknowledgements: We thank Dr. F. Winkler for many stimulating discussions, Dr. S.E. Halford and Dr. B. Connolly for unpublished data, and R. Mull-Grotefend for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Pi 122/5-2, Pi 122/2-4, Mu 465/11-7) and the Fonds der Chemischen Industrie.

REFERENCES

- [1] Harrison, S.C. and Aggarwal, H.K. (1990) *Annu. Rev. Biochem.* 59, 933-969.
- [2] Steitz, T.A. (1990) *Quart. Rev. Biophys.* 23, 205-280.
- [3] Freemont, P.S., Lane, A.N. and Sanderson, M.R. (1991) *Biochem. J.* 278, 1-23.
- [4] Hedgepeth, J., Goodman, H.M. and Boyer, H.W. (1972) *Proc. Natl. Acad. Sci. USA* 69, 3448-3452.

- [5] Polinsky, B., Greene, P., Garfin, D.E., McCarthy, B.J., Goodman, H.M. and Boyer, H.W. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3310-3314.
- [6] Kholmina, G.V., Rebentish, B.A., Skoblov, Y.S., Mironov, A.A., Yanovskii, Y., Kozlov, Y.I., Glatman, L.I., Moroz, A.E. and Debabov, V.G. (1980) *Dokl. Akad. Nauk. SSSR* 253, 495-497.
- [7] Schildkraut, I., Banner, C.D., Rhodes, C.S. and Parekh, S. (1984) *Gene* 27, 327-329.
- [8] Kim, Y., Grable, J.C., Love, R., Greene, P.J. and Rosenberg, J.M. (1990) *Science* 249, 1307-1309.
- [9] Winkler, F.K. (1992) *Curr. Opin. Struct. Biol.* 2, 93-99.
- [10] Taylor, J.D., Badcoe, I.G., Clarke, A.R. and Halford, S.E. (1991) *Biochemistry* 30, 8743-8753.
- [11] Thielking, V., Selent, U., Köhler, E., Landgraf, A., Wolfes, H., Alves, J. and Pingoud, A. (1992) *Biochemistry* (in press).
- [12] Wolfes, H., Alves, J., Fließ, A., Geiger, R. and Pingoud, A. (1986) *Nucleic Acids Res.* 14, 9063-9080.
- [13] Wright, D.J., King, K. and Modrich, P. (1989) *J. Biol. Chem.* 264, 11816-11821.
- [14] King, K., Benkovic, S.J. and Modrich, P. (1989) *J. Biol. Chem.* 264, 11807-11815.
- [15] Thielking, V., Selent, U., Köhler, E., Wolfes, H., Pieper, U., Geiger, R., Urbanke, C., Winkler, F.K. and Pingoud, A. (1991) *Biochemistry* 30, 6416-6422.
- [16] Selent, U., Rüter, T., Köhler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschläger, T., Wolfes, H., Peters, F. and Pingoud, A. (1992) *Biochemistry* (in press).
- [17] Conolly, B.A., Eckstein, F. and Pingoud, A. (1984) *J. Biol. Chem.* 259, 10760-10763.
- [18] Grasby, J., Connolly, B. and Eckstein, F. (in preparation).
- [19] Rosenberg, J.M. (1991) *Curr. Opin. Struct. Biol.* 1, 104-113.
- [20] Freemont, P.S., Friedman, J.M., Beese, L.S., Sanderson, M.R. and Steitz, T.A. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8924-8928.
- [21] Mullen, G.P., Serpersu, E.H., Ferrin, L.J., Loeb, L.A. and Mildvan, A.S. (1990) *J. Biol. Chem.* 265, 14327-14334.
- [22] Beese, L.S. and Steitz, T.A. (1991) *EMBO J.* 10, 25-33.
- [23] Han, H., Rifkind, J.M. and Mildvan, A.S. (1991) *Biochemistry* 30, 11104-11108.
- [24] Clark, M., Cramer, R.D. and Van Opdenbosch, N. (1989) *J. Computational Chem.* 10, 982-1012.
- [25] Lesser, D.R., Kurpiewski, M.R. and Jen-Jacobson, L. (1990) *Science* 250, 776-786.
- [26] Taylor, R.S. and Diebler, H. (1976) *Bioinorg. Chem.* 6, 247-254.
- [27] Basolo, F. and Pearson, R.G. (1967) *Mechanisms of Inorganic Reactions*, John Wiley, New York.
- [28] Nakamura, H., Oda, Y., Iwai, S., Inoue, H., Ohtsuka, E., Kanaya, S., Kimura, S., Katsuda, C., Katayanagi, K., Morikawa, K., Miyashiro, H. and Ikehara, M. (1991) *Proc. Natl. Acad. Sci. USA* 88, 11535-11539.
- [29] Vermonte, C.L.M., Vipond, I.B. and Halford, S.E. (1992) *Biochemistry* (in press).
- [30] McSwiggen, J.A. and Cech, T.R. (1989) *Science* 244, 679-683.
- [31] Dahm, S.C. and Uhlenbeck, O.C. (1991) *Biochemistry* 30, 9464-9469.