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Site directed mutagenesis experiments suggest that Glu 111, Glu 144 and Arg 145 are essential for endonucleolytic activity of EcoRI

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ABSTRACT

We have constructed a plasmid (pRIF 309+) carrying the EcoRI restriction endonuclease gene and the f1 origin of replication. Upon transformation of this plasmid into E.coli and infection with bacteriophage f1 single stranded plasmids are produced which can be used for sequencing and site directed mutagenesis. Using this single stranded DNA and synthetic oligodeoxynucleotides we have introduced point mutations at defined positions of the EcoRI gene. Since in pRIF309+ the EcoRI gene is under the control of the p_L-promoter, high level expression of the mutated EcoRI gene could be obtained upon induction. Mutant EcoRI enzymes were purified to homogeneity and characterized in structural and functional terms. Our results demonstrate that the Glu 111 -> Gln, Glu 144 -> Gln and Arg 145 -> Lys -mutants adopt a very similar conformation as the wild type enzyme, but have by two orders of magnitude smaller specific activities than the wild type enzyme, mainly due to a reduction of the V_{max}-value.

INTRODUCTION

The type II restriction endonuclease EcoRI is one of over 500 enzymes that recognize a specific DNA sequence and cleave the DNA within or in the immediate vicinity of this sequence. Restriction enzymes have proven to be indispensible tools for the analysis, preparation and in vitro recombination of DNA. Because of their importance many structural and mechanistic studies dealing with restriction endonucleases have been carried out, in particular for EcoRI, the so far best studied type II restriction endonuclease (for review cf. (1)). Few of these studies were directed towards the identification of amino acid residues of importance for the specific binding and cleavage of the DNA substrate. It was shown for EcoRI that lysine (2), glutamic acid (3) and histidine residues (4) are essential for the activity of this enzyme, since their chemical modification inactivates the

enzyme. It was not shown in these investigations, however, which of the many lysine, glutamic acid and histidine residues contained in EcoRI are the essential ones, nor whether the binding and/or the catalytic activity of EcoRI was affected by the chemical modification. More recently, it was shown by an immunological study (5) and by cross-linking experiments (6) that the region comprising residues 137 to 157 of the EcoRI molecule is involved in the binding of DNA. Furthermore, in a preliminary presentation (7) of a 3 Å structure of a DNA-EcoRI endonuclease recognition complex evidence was given which suggests that EcoRI interacts with its substrate via hydrogen bonds between glutamic acid 144 and arginine residues 145 and 200 on one side and the guanine and adenine bases of the target sequence GAATTC on the other side. The hydrolytically active site according to this analysis is supposed to be located in a cleft which is formed by polypeptide loops involving residues from the N-terminal half of the EcoRI molecule. Based on these structural informations from studies in solution as well as from x-ray crystallography we have begun with site directed mutagenesis experiments in order to identify amino acid residues involved in the specific recognition of the substrate and the catalysis of cleavage.

We demonstrate in the present paper that Glu 111, Glu 144 and Arg 145 are essential for the enzymatic activity, since their replacement by glutamine and lysine, resp., leads to EcoRI mutants with drastically lowered catalytic activity. Since, furthermore, the mutations do not affect the secondary structure or gross conformation of the enzyme, we conclude that Glu 111, Glu 144 and Arg 145 are involved in specific binding and/or catalysis.

METHODS

Oligodeoxynucleotide synthesis

Mismatch primers for site directed mutagenesis and primers for DNA sequencing were synthesized on a bench synthesizer (8) via established phosphotriester chemistry as described recently (9) or on the Biosearch model 8600 DNA synthesizer using cyanoethyl-phosphoramidites supplied by the manufacturer. The crude reaction products, 5'-dimethoxytrityl-oligodeoxynucleotides, were

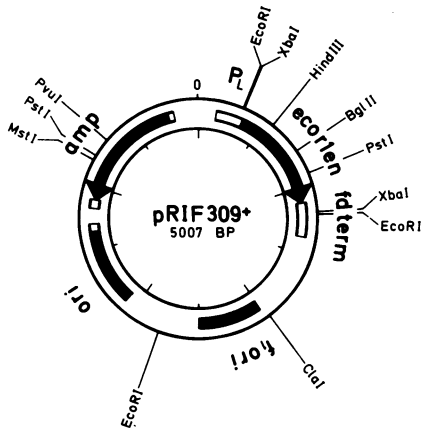

Fig.1: Map of pRIF309+

purified by preparative HPLC on a 2.5 x 25 cm Lichrosorb RP 18 (Merck) column at a flow-rate of 4 ml/min using a 120 min gradient from 10 % acetonitrile to 50 % acetonitrile in 0.1 M triethylammonium acetate pH 7.0. The purified oligodeoxynucleotides were lyophilized and deprotected in 80 % acetic acid, lyophilized again, and extracted three times with diethyl ether. The purity of all oligodeoxynuceotides were checked by HPLC on wide pore PEI (Baker) column at a flow rate of 0.4 ml/min using a 60 min gradient from 1 mM to 210 mM K-phosphate pH 6.3 in 60 % (v/v) formamide (10 - 13).

Construction of a vector for overexpressing, mutating and sequencing the EcoRI gene

The expression plasmid pEcoR309 (14) with the EcoRI endonuclease gene under the control of the p_L promoter of bacteriophage as well as the plasmid pSSVII which contains a 1.2 kbp fragment comprising the intergeneic region of bacteriophage f1 cloned into the EcoRI site of pBR322 were kindly given to us by Dr. M. Zabeau (Gent). The 1.2 kbp fragment was ligated into the single Tth111 I restriction site of pEcoR309 between the EcoRI gene and the origin of replication to give the plasmids pRIF309+ and pRIF309- (Fig. 1). In pRIF309+ the orientation of the intergenic region is such that it directs the production of the single strand of the plasmid DNA which contains the sense strand of the

EcoRI endonuclease gene. Similar as with the parent plasmid pEcoR309 pRIF309+ when transformed into E.coli codes for a EcoRI gene which is totally repressed in the presence of the CI repressor of bacteriophage λ or in the presence of the thermosensitive repressor CI857 at 30°C. Consequently, there is no need for the EcoRI methylase to be present in the cells to prevent cleavage of the chromosomal DNA by transiently expressed endonuclease. A temperature shift to 42°C in the presence of repressor CI857 leads to an overproduction of the EcoRI endonuclease yielding approx. 10% of total cellular protein. Upon superinfection of F'-strains carrying pRIF309+ with bacteriophage f1 single stranded plasmid DNA is packaged and excreted into the culture medium like bacteriophage f1 DNA, similary as described for the pEMBl plasmid family (15). In the early phase of infection there is about 4 times more plasmid DNA excreted than bacteriophage f1 DNA. This DNA can be isolated like M13 DNA and can be used for dideoxy sequencing and site directed mutagenesis.

Site directed mutagenesis

Site directed mutagenesis was carried out on single stranded plasmid DNA using protocols established for M13 bacteriophage DNA (16). For this purpose single stranded plasmid DNA was produced as follows. Clones of interest were grown in 3 ml LB/amp overnight. 400 µl were taken to inoculate 20 ml LB/amp in an Erlenmeyer flask which was kept at 37°C for 3 hours, until a turbidity of approx. 0.3 A_{600nm} was reached. Then the cells were infected with 5 x 10^9 pfu f1 phages and incubation was continued for another 3 hours. After centrifugation at 24000 g for 20 min PEG 6000 and NaCl were added to the supernatant to give 5% PEG 6000, 0.6 M NaCl and the resulting mixture was incubated for 15 min at room temperature. Centrifugation at 8000 g for 20 min yielded a pellet which was suspended in 600 µl 1 mM Tris/HCl, pH 8.0, 0.1 mM EDTA and extracted with 400 µl distilled phenol. To the aqueous phase 100 µl 5 M $NaClO_4$ and 500 µl isopropanol were added. The precipitate was collected by centrifugation and dried in a desiccator. The DNA was dissolved in 300 µl 0.3 M Na-acetate and precipitated with 900 µl ethanol. After 2 hours at -20°C the turbid solution was centrifuged, the pellet was dried

and redissolved in 100 µl 1 mM Tris/HCl, pH 8.0, 0.1 mM EDTA. 5 µg of single stranded pRIF309+ in 25 µl annealing buffer (150 mM NaCl, 150 mM Tris/HCl, pH 8) were mixed with 1.5 mA_{260nm} phosphorylated oligodeoxynucleotide in 13 µl annealing buffer. This mixture was heated to 70°C for 15 min and was allowed to cool to room temperature over a period of 3 hours. Subsequently, 10 µl 100 mM $MgCl_2$, 1 µl 100 mM ATP, 3 µl 10 mM dATP, 3 µl 10 mM dCTP, 3 µl 10 mM dGTP, 3 µl 10 mM dTTP, 25 µl distilled water, 12 U of DNA polymerase I (Klenow fragment) and 4 U of T4 DNA ligase were added.

This reaction mixture was incubated at 16°C overnight. 100 µl competent E.coli LK111(λ lysogen) cells harvested during logarithmic growth and stored at -80°C in a solution of 0.1 M $CaCl_2$ and 50% glycerol (v/v) were thawed on ice, 5 µl of the Klenow reaction mixture were added. The transformation mixture was incubated for 5 min at 0°C and then for another 5 minutes at 41°C. After addition of 1 ml LB-medium incubation was continued for 30 min at 37°C. 300 µl of this solution was plated onto LB agar plates containing 100 µg/ml ampicillin (LB/amp). The plates were left in an incubator at 37°C overnight. More than 500 colonies of transformants on each plate were obtained. 48 colonies were picked randomly from each plate and inoculated in 150 µl LB/amp in the wells of a microtiter plate. This plate was incubated at 37°C for 16 hours. A nitrocellulose filter was placed onto an LB/amp agar plate, colonies grown in the wells were spotted onto the nitrocellulose filter. They were incubated for 12 hours at 37°C. The cells grown on the nitrocellulose were broken up by treating them twice with 0.5 M NaOH for 5 min. After drying they were subsequently wetted twice for 5 min with 1 M Tris/HCl, pH 7.4 , 1.5 M NaCl, and then with 0.5 M Tris/HCl, pH 7.4, 0.3 M NaCl. The fixation of the DNA to the support was performed by incubation at 80°C for 2 hours. In order to screen the transformants for mutants the nitrocellulose filters were incubated with 5 ml of a 1:1 mixture of 10 x Denhardts solution and 6 x SSC in a Petri dish for 15 min at room temperature. The solution was decanted, 3 ml of the prehybridisation mix with the radioactively labelled mutagenesis primer were added to the nitrocellulose filter which were then incubated at 70°C for 5 min. The mixture

was left at room temperature for 30 minutes. The filters were washed with 6 x SSC at room temperature and at 60°C. The filters were wrapped in Saran wrap and exposed to Kodak XAR 5 film for 2 days. From mutant clones identified by autoradiography plasmid DNA was isolated and used for the transformation of E.coli LK111(λ lysogen) cells. Ampicillin resistent colonies obtained after incubation overnight at 37°C were inoculated on microtitre plates, grown on nitrocellulose filters and subjected to a second screening procedure. After autoradiography 4 positive clones from each filter were chosen for the sequencing reaction.

Sequencing

For characterization of the mutant clones the relevant parts of the EcoRI genes were sequenced by the dideoxy method (17,18). In one instance, namely for the Gln 111 mutant all of the gene was sequenced. For this purpose single stranded plasmid DNA was produced as described above. 2 µl 0.1 M Tris/HCl, pH 7.4, 0.1 M $MgCl_2$, 0.5 M NaCl and 5 µl of the single stranded DNA solution containing approx. 1 µg DNA were mixed with 2 µl of a solution containing approx. 5 ng of the appropriate sequencing primer. This solution was diluted with distilled water to a volume of 17 µl. The mixture was incubated at 95°C for 5 min and allowed to cool down to room temperature. This solution was divided into portions of 4 µl. To each tube 2 µl dNTP, 1.5 µl ddNTP (A,C,G or T), 1 µl ^{35}S α dATP (= 1.5 µCi), 1 µl DNA-polymerase large fragment (1.25 u) were mixed and incubated at room temperature for 30 min. 2 µl of the dNTP chase solution were added and incubated at room temperature for 50 min. The reaction was stopped with 40 µl stop solution and 200 µl ethanol, and then left at -20°C overnight. After removing the liquid phase the pellets were washed with 70% ethanol, dissolved in 3 µl formamide, 20 mM EDTA and 0.1 % bromphenol blue as marker dye and electrophoresed on 6% PAGE-gels containing 8 M urea. The gels were exposed for 2 days at -80°C with Kodak XAR 5 films.

Fermentation

For expression of the mutant EcoRI genes the mutated pRIF309+ DNA was prepared and employed for transformation of E.coli TGE 900 (vide infra). For this purpose E.coli LK111(λ lysogen) containing the plasmid pRIF309+ were grown overnight in a 1 ml cul-

ture, harvested by centrifugation and resuspended in 200 µl 50 mM Tris/HCl, pH 8.0, 50 mM EDTA, 15% (w/v) sucrose and 10 µl lysozyme (12 mg/ml). After incubation on ice for 20 min 800 µl distilled water was added and the solution was heated to 75°C for 2 min and 50 µl phenol was added. After centrifugation at 40000 g for 20 min the aqueous phase was carefully removed and extracted with 200 µl chloroform-isoamylalcohol 24:1 (v/v). DNA was precipitated with 100 µl 5 M $NaClO_4$ and 500 µl isopropanol. The pellet was collected by centrifugation at 15000 g for 15 min. The product was dried in a desiccator and redissolved in 50 µl 1 mM Tris/HCl, pH 8.0, 0.1 mM EDTA. 5 µl of this preparation were used for transformation of E.coli TGE 900. Strain TGE 900 F^-,su^-, ilv^-, bio (ZcI857 delta Bam delta HI))(kindly given to us by Dr. A. Balland, Strasbourg) which produces a temperature sensitive repressor was used as host for overexpression of the EcoRI gene. The transformation procedure was the same as described before. The mutants were grown in 8 l M9 medium at 28°C for 3.5 hours to give a turbidity of approx. 0.7 A_{600nm}. Then the temperature was shifted to 42°C for 3 hours. Cells were harvested by centrifugation and washed with 30 mM K-phosphate, pH 7.2, 1 mM EDTA, 0.1 mM DTE, 0.1 M NaCl. After a second centrifugation they were resuspended in 30 mM K-phosphate, pH 7.2, 0.1 mM DTE, 1 M NaCl and stored at -20°.

Purification of mutant EcoRI endonucleases

The purification of EcoRI from the overproducing E.coli TGE900 strains was carried out similary as described recently (5,19). Approx. 20 - 40 g wet cell paste, the typical yield of a 8 l fermentation, were suspended in 150 ml buffer A (0.03 M K-phosphate, pH 7.2, 1 mM EDTA, 0.1 mM DTE, and 0.01% (w/v) Lubrol) containing 1 M NaCl. Cells were broken up by sonication in a MSE MK 2 sonifier at 150 W for 15 min with intermittant cooling to keep the temperature of the suspension below 10°C. All subsequent steps were carried out at 4°C. Cell debris was removed by centrifugation at 30000 g for 20 min. The supernatant was dialyzed overnight against 5 l of buffer A containing 0.2 M NaCl and then loaded onto a phosphocellulose column (2.5 x 20 cm) equilibrated with buffer A containing 0.2 M NaCl. After rinsing the column with equilibration buffer elution was carried out by a

linear gradient of 2 x 1.5 l 0.2 - 1.0 M NaCl in buffer A. Fractions containing EcoRI as determined by SDS-PAGE (vide infra) were pooled and applied to a hydroxyapatite column (2.5 x 10cm) equilibrated with buffer A containing 0.5 M NaCl. After rinsing the column with equilibration buffer elution was carried out by a linear gradient of 2 x 1.5 l of 0 - 0.57 M K-phosphate in buffer A containing 0.5 M NaCl. Fractions containing EcoRI were pooled and concentrated by dialysis against 1 l of buffer A containing 20% PEG 10000 and 0.2 M NaCl, and then loaded onto a AcA44 (LKB) column (2.5 x 200 cm) equilibrated with buffer B (0.05 M Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 m M DTE and 0.01% (w/v) Lubrol) containing 0.2 M NaCl. Fractions containing EcoRI were pooled, concentrated by dialysis against buffer B containing 0.2 M NaCl and 10% PEG 10000. The concentrated sample was diluted with 3 volumes water and loaded onto a DE52 (Whatman) column (0.4 x 2 cm) equilibrated with buffer A. The column was rinsed with buffer A and EcoRI eluted with buffer A containing 0.3 M NaCl. Fractions containing EcoRI were pooled, diluted with 4 volumes glycerol and stored at -30° C. This purification scheme yields within 6 days EcoRI that is at least 95% pure. The yield was approx. 5 A_{280nm} units. The EcoRI endonuclease mutants described here show only very reduced enzymatic activity. The isolation procedure, therefore, had to be monitored by SDS PAGE. For electrophoresis, typically 10 µg bovine serum albumine were added to 500 µl aliquots in order to prevent irreversible aggregation of EcoRI during the subsequent precipitation step. EcoRI and bovine serum albumine were coprecipitated with 50 mg trichloroacetic acid. The precipitate was dissolved in 20 µl loading buffer (0.2 M Tris/HCl, pH 8.6, 3% (w/v) SDS, 40% (v/v) glycerol, 3% (v/v) β-mercaptoethanol, 0.01 (w/v) bromphenol blue) and analyzed on 17.5% polyacrylamide gels in the presence of SDS.

BrCN cleavage of mutant EcoRI endonucleases

In order to verify that the protein isolated by a procedure which was only monitored by SDS PAGE was indeed mutant EcoRI endonuclease BrCN cleavage was carried out as described (5,6).

Gel filtration

In order to find out, whether mutant EcoRI endonuclease mole-

cules like wild type EcoRI form dimers or tetramers at higher concentration (20), we have carried out analytical gel filtration experiments. For this purpose a 50 µl sample containing approx. 5 µg of mutant EcoRI and 0.5 µg of wild type EcoRI were loaded onto an AcA44 (LKB) column (0.4 x 31cm), equilibrated with buffer A containing 0.2 M NaCl. Fractions comprising 10 drops (approx. 220 µl) were collected and analyzed for EcoRI activity by a DNA cleavage assay as well as for protein composition by SDS PAGE. Coincidence of EcoRI activity which is mainly due to wild type EcoRI and protein content which predominantly is given by the EcoRI mutant under investigation would strongly suggest that mutant EcoRI like wild type EcoRI is dimeric under the conditions given and, furthermore, adopts a similar globular structure.

Circular dichroism spectroscopy

Circular dichroism spectra of wild type and mutant EcoRI were recorded in order to find out whether the mutations introduced into EcoRI affect the secondary structure of this protein. All measurements were carried out at ambient temperature in cylindrical cuvettes of 0.05 cm pathlength on a R.J. Mark III dichrograph (Jobin-Yvon). The spectra were analyzed for α-helix, β-pleated sheat and remainder structure as described previously (21).

DNA cleavage experiments

Bacteriophage λ DNA or plasmid pUC8 DNA was incubated with mutant or wild type EcoRI in 0.02 M Tris/HCl, pH 7.2, 0.05 M NaCl, and 0.01 M $MgCl_2$ at 37°C. The reaction was stopped by adding 5 µl of a solution containing 0.25 M EDTA, 0.2% (w/v) SDS, 25% (w/v) sucrose and 0.1% (w/v) bromphenol blue to a 10 µl aliquot. Reaction products were separated on 1% or 2% agarose gels. The cleavage of λ DNA was analyzed in order to find out, whether EcoRI mutants produce the same cleavage pattern as wild type EcoRI and in order to determine the specific activities of the mutant enzymes. 1 U is definded as the amount of enzyme required to produce a complete digest of 1 µg DNA in 60 min in a total reaction volume of 15 µl. The cleavage of supercoiled pUC8 DNA was analysed in a quantitative manner. For this purpose the ethidium bromide stained gels were photographed, the negatives

were scanned with a LKB 2202 Ultroscan laser densitomter, the scans digitized and numerically integrated. In order to derive K_M and V_{max} values from these data (v_o vs. $c_{substrate}$) a computerized procedure was used (courtesy of Dr. F. Peters, Hannover).

RESULTS

We have constructed a plasmid (pRIF309+) which harbours the EcoRI gene the sequence of which was published recently (22,23) and confirmed by us. It contains the fd origin of replication which allows single stranded plasmid DNA to be produced upon infection of E.coli cells by a f1 helper phage. This DNA can be isolated like M13 DNA and can be used for dideoxy sequencing and site directed mutagenesis. Using the single stranded plasmid DNA and the following synthetic oligodeoxynucleotides

I d(CTTGTTGCTCAAGCCAAACA) (Glu111- Gln)

II d(AATGCTATCCAAAGATCTC) (Glu144- Gln)

III d(CTATCGAAAAATCTCATAA) (Arg145- Lys)

which carry a single mismatch (underlined) with respect to the sequence to be mutated we have introduced point mutations into the EcoRI gene. Annealing, polymerization and ligation was carried out following established protocols. After transformation into E.coli LK111 of pRIF309+ carrying the mismatch , positive clones were identified by colony hybridization with the radioactively labelled mismatch oligonucleotide and characterized by sequencing the EcoRI gene or relevant portions of it (results not shown). Mutant EcoRI enzymes were obtained by transformation into E.coli TGE900 of pRIF309+ carrying the mutation . Since in pRIF309+ the EcoRI gene is under control of the p_L promoter, high level expression is obtained upon induction. Mutant enzymes were isolated in homogeneous form (> 95% pure) by phosphocellulose, hydroxyapatite, AcA44 and DE52 chromatography which had to be monitored by SDS PAGE because of the low specific activity of the mutant enzymes. The EcoRI mutants were shown by BrCN digestion followed by SDS PAGE to produce the same peptide pattern as wild type EcoRI confirming that we have isolated authentic mutants of EcoRI and not just a protein of 31 kd molecular

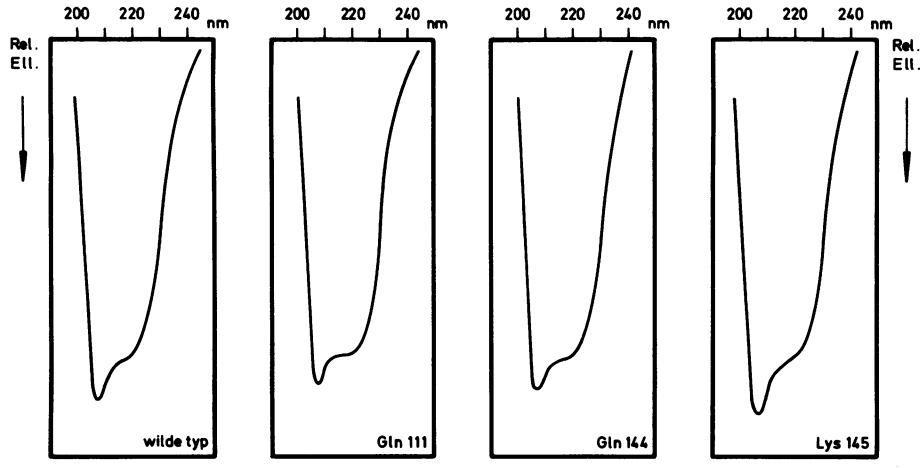

Fig.2: Circular dichroism spectra of wild type and mutant EcoRI

weight (results not shown). The EcoRI mutants which we have obtained form dimers of identical subunits like wild type EcoRI under the conditions used. This was established by analytical gel filtration experiments which showed that wild type and mutant EcoRI have the same elution volume (results not shown). Since, furthermore, circular dichroism spectra of mutant and wild type EcoRI enzymes are very similar (Fig. 2), we conclude that the mutations which we have introduced have not affected the gross structural features of EcoRI. The enzymatic activity of the mutant enzymes was determined with bacteriophage DNA as substrate. Under normal buffer conditions, i.e. 20 mM Tris/HCl, pH 7.2, 50 mM NaCl, 10 mM $MgCl_2$, DNA is cleaved at the canonical sites, albeit with considerably reduced specific activity when compared to the wild type enzyme, as shown in table 1.

This is also apparent from the kinetics of cleavage of pUC8 DNA (Fig. 3). The time course of cleavage of pUC8 DNA which has

Table 1: Specific activities (U/mg) of wild type and mutant EcoRI enzymes

wt	Gln 111	Gln 144	Lys 145
3.5×10^6	9.7×10^4	4.4×10^4	6.3×10^4

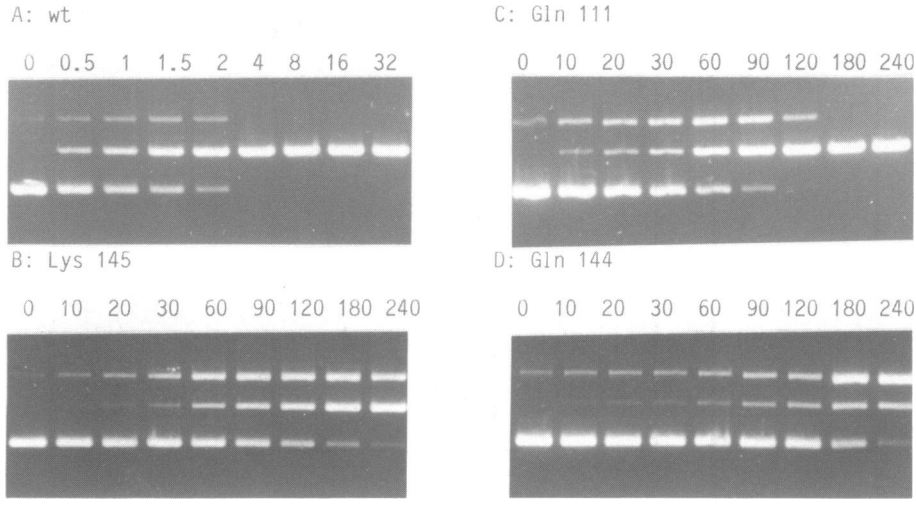

Fig. 3: Cleavage of pUC8 DNA by wild type and mutant EcoRI enzymes. 99 nM pUC8 DNA and 18 nM enzyme were incubated at 37°C. At the times indicated aliquots were withdrawn, the reaction was stopped, and the products of the reaction analyzed by electrophoresis on 2 % agarose gels. A: wild type enzyme, time = 0, 0.5, 1, 2, 4, 8, 16, 32 min. B, C, D: Lys 145, Gln 111, Gln 144 mutants, time = 0, 10, 20, 30, 60, 90, 120, 180, 240 min. Note the different time scales of the wild type and mutant EcoRI catalyzed cleavage reactions. Cleavage experiments as shown here were used for the determination of K_M and V_{max} values.

only one site for EcoRI was analyzed in terms of K_M and V_{max} values. Table 2 gives a compilation of the K_M and V_{max} values obtained for the cleavage of pUC8 DNA by the wild type and mutant enzymes.

Table 2: K_M (nM) and V_{max} (min^{-1}) values of wild type and mutant EcoRI enzymes

	wt	Gln 111	Gln 144	Lys 145
K_M	50	95	120	105
V_{max}	19	0.3	0.2	0.3

DISCUSSION

EcoRI is by far the best studied restriction endonuclease, both with respect to structure and mechanism. It is known that EcoRI attacks its substrate by first binding to DNA in an non-specific manner (2,21,24,25). This binding is mediated mostly through electrostatic interactions and is accompanied by a conformational change of the enzyme (21). Target site location involves multiple association-dissociation events as well as linear diffusion (26,27), whose effectiveness very much depends on the buffer conditions (28,29). The recognition of the cleavage site must be very complex: from studies with oligodeoxynucleotides one knows that the strengh of binding increases by two to five orders of magnitude depending on the size of the oligodeoxynucleotide (21,30,31). Non-electrostatic interactions presumably are of prime importance for the recognition of the individual bases comprising the cleavage site (30,32). This recognition involves deformation of the DNA and conformational changes of the enzyme (33,35) which together produce in particular the juxtaposition of hydrogen bond donors and acceptors of the enzyme and the substrate necessary for specific complex formation (36). While Mg^{2+}- ions are not necessary for strong ("specific") binding, they or other divalent cations are neeeded for cleavage to occur (37,38). The role of Mg^{2+} in the catalytic process is not yet understood. Since in the EcoRI molecule recognition site and cleavage site are spatially separated (7), the recognition process must eventually trigger a conformational change which activates the catalytic centre.

Relating structural details and mechanistic features for this enzyme is currently pursued in several laboratories. Site directed mutagenesis undoubtly is one of the most powerful techniques for such studies, as has been demonstrated successfully for the tyrosyl tRNA synthetase (e.g. 9,40). We have now begun to identify by site directed mutagenesis experiments individual amino acid residues which are involved in specific binding and catalysis: In this paper we have presented details of our methodology and the results of a few site directed mutagenesis experiments.

In planning our experiments we relied heavily on two sets of informations:

I. It was shown by Woodhead and Malcolm that the chemical modification of a single glutamic acid residue is responsible for the inactivation of the enzyme by a carbodiimide reagent (3). The possibility, however, was not ruled out, that two or more glutamic acid residues react with the carbodiimide at the same rate and that modification of any one is sufficient to inactivate the enzyme.

II. Rosenberg and colleagues have solved the three dimensional structure of the co-crystalline recognition complex between EcoRI and the cognate oligodeoxynucleotide d(TCGCGAATTCGCG) at 3 Å resolution (7) which provides information as to the location of amino acid side chains which might be involved in the binding and cleavage of the substrate. According to this structure, arginine (Arg 145) and glutamic acid (Glu 144) side chains of each EcoRI subunit form bidentate hydrogen bonds to the two adenine residues of the EcoRI recognition site, each adenine being hydrogen bonded to an arginine and a glutamic acid side chain. From our own previous structural studies in solution we know that the region between residue no. 137 and 157 of the enzyme is directly involved in the binding of the substrate (5,6), in agreement with the above assignment. According to Rosenberg et al the hydrolytically active site is located in the N-terminal half. From an inspection of their schematic backbone drawing it seemed to us that Glu 111, although in the crystal structure not making contact with the DNA, could come sufficiently close to the DNA backbone to be involved in the catalysis of cleavage. In order to investigate whether Glu 111, Glu 144 and Arg 145 are involved in the binding and cleavage of DNA by the EcoRI endonuclease, we have by site directed mutagenesis replaced Glu 111 by Gln, Glu 144 by Gln and Arg 145 by Lys, purified the three mutant proteins to homogeneity, characterized them by BrCN digestion and subjected them to a variety of structural and functional assays. The gross structural features, including the dimeric structure, are unaltered by the single and conservative amino acid substitutions. Circular dichroism spectroscopy shows that the mutant and wild type proteins have within the limits of

error identical secondary structure. While, by these criteria, the mutant and wild type EcoRI endonuclease have very similar overall structures, they behave very differently when tested for enzymatic activity in a DNA cleavage assay. The mutants have lost most of their endonucleolytic activity, the specific activity being reduced by two orders of magnitude. The analysis of the steady state kinetics of DNA cleavage by the wild type and the mutant EcoRI enzymes shows that the Gln 111, Gln 144 and Lys 145 mutants have a slightly higher K_M with respect to cleavage of supercoiled pUC8 DNA than wild type EcoRI. For all three mutants the reduction in the specific activity is mainly due to a decrease in V_{max} as compared to the wild type enzyme.

There are several possible ways in which glutamic acid residues could play a role in the catalytic action of EcoRI as pointed out by Woodhead & Malcolm (3): The carboxyl group could bind the essential divalent cation. It could also be involved in a charge relay system, a particular attractive possibility in the light of the recent suggestion as to a mechanism of action of DNase I (41) which involves a Glu-His-H_2O relay or it could be responsible for recognition of individual bases within the recognition sequence as proposed for Glu 144 (7). In addition, the possibility must be considered that a glutamic acid residue is involved in stabilizing a transient conformation of the enzyme substrate complex which is necessary for catalysis to occur. Our results show that indeed glutamic acid residues are essential for the EcoRI catalyzed reaction, Glu 111 possibly being involved directly or indirectly in catalysis and Glu 144 presumably being necessary for specific binding of the substrate.

Arginine residues might be involved in nonspecific interaction with DNA by forming ion pairs with the negatively charged phosphodiester backbone (31). They could also be responsible for specific recognition of individual bases within a recognition sequence, as proposed for Arg 145 and Arg 200 (7). We have shown here that Arg 145 is essential for high enzymatic activity, in the light of the X-ray structure most likely because of its involvement in specific binding of DNA.

It is surprising to note that the Gln 144 and Lys 145 mutants

which presumably are impaired in specific interactions between enzyme and substrate have K_M values with respect to cleavage of DNA that are only slightly higher than that of the wild type EcoRI. This is not what one would have expected from a linear relationship between the strength of binding and the number of hydrogen bonds formed between enzyme and substrate. It rather reflects a complex synergism of indiviual interactions which take place during the recognition process. Furthermore, K_M values cannot simply be interpreted as K_{Diss} values of the enzyme substrate complex. This is particularly true for EcoRI, for which non-specific binding of the enzyme to DNA and linear diffusion along the DNA will strongly influence the K_M value (24,27). On the other hand the Gln 144 and Lys 145 mutant have a much smaller V_{max} for the cleavage of DNA than the wild type enzyme. Again this is not what one would have expected for mutants unimpaired in their catalytic center. However, it is conceivable that EcoRI mutants deficient in the recognition site have a drastically lowered V_{max}, as we have found, if one assumes that the catalytic site is allosterically tightly linked to the recognition site.

It remains to be established, whether the mutant enzymes show a decreased specificity with respect to the cleavage of DNA at the canonical sequence. To this end we are currently engaged in a detailed thermodynamic and kinetic analysis of the binding of the wild type and the mutant enzymes to synthetic oligodeoxynucleotides comprising the recognition site, a closely related site or an unrelated site. For the same purpose we have started an analysis of the accuracy of cleavage under normal buffer conditions. Preliminary experiments show that the Gln 144 and Lys 145 mutants are not drastically impaired in their specificity.

In conclusion, although we have identified two glutamic acid and one arginine residue in EcoRI to be essential for the EcoRI catalyzed cleavage of DNA, the identification of their role in the enzymatic function of EcoRI is preliminary. More precise structural data and many more data from site directed mutagenesis experiments are needed for the understanding of the interplay of structure and function of this restriction endonuclease.

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