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Genetic Engineering of *Eco*RI Mutants with Altered Amino Acid Residues in the DNA Binding Site: Physicochemical Investigations Give Evidence for an Altered Monomer/Dimer Equilibrium for the Gln144Lys145 and Gln144Lys145Lys200 Mutants†

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ABSTRACT: We have genetically engineered the Arg200 → Lys mutant, the Glu144Arg145 → GlnLys double mutant, and the Glu144Arg145Arg200 → GlnLysLys triple mutant of the *Eco*RI endonuclease in extension of previously published work on site-directed mutagenesis of the *Eco*RI endonuclease in which Glu144 had been exchanged for Gln and Arg145 for Lys [Wolfes et al. (1986) *Nucleic Acids Res. 14*, 9063]. All these mutants carry modifications in the DNA binding site. Mutant *Eco*RI proteins were purified to homogeneity and characterized by physicochemical techniques. All mutants have a very similar secondary structure composition. However, whereas the Lys200 mutant is not impaired in its capacity to form a dimer, the Gln144Lys145 and Gln144Lys145Lys200 mutants have a very much decreased propensity to form a dimer or tetramer depending on concentration as shown by gel filtration and analytical ultracentrifugation. This finding may explain the results of isoelectric focusing experiments which show that these two mutants have a considerably more basic p*I* than expected for a protein in which an acidic amino acid was replaced by a neutral one. Furthermore, while wild-type *Eco*RI and the Lys200 mutant are denatured in an irreversible manner upon heating to 60 °C, the thermal denaturation process as shown by circular dichroism spectroscopy is fully reversible with the Gln144Lys145 double mutant and the Gln144Lys145Lys200 triple mutant. All *Eco*RI endonuclease mutants described here have a residual enzymatic activity with wild-type specificity, since *Escherichia coli* cells overexpressing the mutant proteins can only survive in the presence of *Eco*RI methylase. The detailed analysis of the enzymatic activity and specificity of the purified mutant proteins is the subject of the accompanying paper [Alves et al. (1989) *Biochemistry* (following paper in this issue)].

It is believed that class II restriction endonucleases like repressors recognize their palindromic target size as dimers composed of identical subunits by forming a symmetrical complex in which both protein subunits are engaged in the same set of interactions with their substrate (Smith, 1979). While considerable progress has been made in elucidating the structural basis for the high specificity of repressors toward their operator sites (Anderson et al., 1981, 1987; Pabo & Lewis, 1982; Schevitz et al., 1985; Lehming et al., 1987), similar detailed knowledge is only beginning to emerge for restriction enzymes, in particular for the *Eco*RI endonuclease (McClarin et al., 1986). The target sites of restriction endonucleases are small, typically four to eight base pairs long, hence considerably smaller than those of repressors (Modrich & Roberts, 1982). Nevertheless, restriction endonucleases

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cleave DNA very specifically within their target site. *Eco*RI and *Eco*RV, e.g., cleave noncanonical sites which differ in one base pair from the canonical site by about 4 orders of magnitude more slowly than their canonical site (Landgraf, 1987).

*Eco*RI is the only enzyme for which a detailed X-ray structure analysis of a cocrystal with an oligonucleotide substrate has been published (McClarin et al., 1986). From the crystal structure several amino acid residues have been identified which are assumed to be responsible for the sequence specificity of *Eco*RI: Glu144, Arg145, and Arg200. We have recently described two mutants of *Eco*RI which have altered amino acid residues in the DNA binding site: Glu144 → Gln and Arg145 → Lys (Wolfes et al., 1986). These two mutants have a similar if not an identical structure as the wild-type enzyme. They show a decreased activity, presumably due to the absence of a hydrogen-bond acceptor, the carboxylate function of Glu144, and of a hydrogen-bond donor, the guanidinium function of Arg145, which both are assumed to be involved in the recognition of the substrate. It was surprising to note that the mutations did not affect the specificity of the enzyme to a measurable extent. Since a third amino acid residue, Arg200, is presumably also involved via hydrogen bonds in specific complex formation with the substrate, we have decided to produce, isolate, and analyze the Arg200 → Lys mutant, in order to find out whether this mutant retains activity and specificity. Glu144, Arg145, and Arg200, according to the X-ray structure analysis, cooperate in the recognition of the substrate. We have, therefore, wanted to produce also the double and triple mutants Glu144Arg145 → GlnLys and Glu144Arg145Arg200 → GlnLysLys and characterize these proteins with respect to their enzymatic function.

In the present paper the site-directed mutagenesis and the isolation of three *Eco*RI mutant proteins are described, as well as the physicochemical characterization of their structural properties. The detailed analysis of the enzymatic activity of these mutants, with respect to both DNA and oligodeoxynucleotide substrates, is dealt with in the accompanying paper (Alves et al., 1989).

Experimental Procedures

Enzymes. *Asu*II was obtained from Anglian Biotechnology; T4 DNA ligase and Klenow polymerase were purchased from Pharmacia; all other enzymes were from Boehringer-Mannheim. Protein standards for isoelectric focusing were obtained from Sigma.

Media. All microbiological media were from GIBCO.

Other Chemicals. Antibiotics were purchased from Sigma. Acrylamide, bis(acrylamide), and agarose (supergrade) were from BRL. Urea was obtained from BDH. All other chemicals were from Merck.

Oligonucleotide Synthesis and Purification. Mismatch primers for site-directed mutagenesis and primers for DNA sequencing were synthesized on a Biosearch Model 8600 DNA synthesizer using β-cyanoethyl phosphoramidites. Protected monomers and tetrazole were from Cruachem, acetonitrile was from Baker, and all other chemicals were from Merck or Fluka. The yield of the crude product obtained from synthesis on a 0.25-μmol scale was about 30 OD^{260} units. Deprotected oligonucleotides were purified by electrophoresis on a 17.5% (w/v) polyacrylamide gel in the presence of 7 M urea. The gel slice containing the desired oligonucleotide as determined by shadow casting was cut out and the oligonucleotide eluted with water overnight at room temperature. Desalting of the oligonucleotide was done on a DE52 cellulose (Whatman) column equilibrated with 0.2 M triethylammonium acetate (TEAA), pH 7.0. Oligonucleotides were eluted with 1 M TEAA, pH 7.0. After lyophilization the oligonucleotides were suspended in 50% (v/v) ethanol, lyophilized again, then dissolved in water.

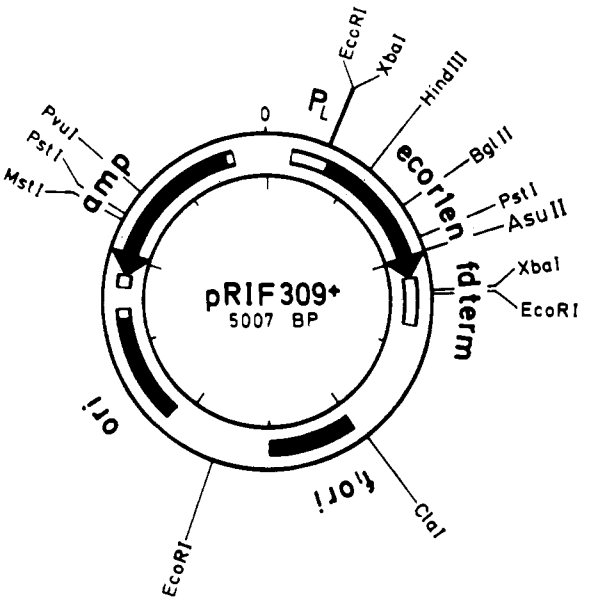

FIGURE 1: Map of pRIF309+. This vector contains the *Eco*RI endonuclease gene under the control of the P_L promoter: by inactivation of the cI repressor, the *Eco*RI gene is efficiently expressed leading to the overproduction of *Eco*RI. The f1 origin of replication after infection with f1 phages allows the production of single-stranded pRIF309+ which can be used for site-directed mutagenesis and sequencing of the *Eco*RI gene.

Bacterial Strains and Plasmid Vectors Used for Mutagenesis, Sequencing, and Overexpression of the EcoRI Endonuclease Gene. The construction of the vector pRIF309+ which we have used for mutagenesis, sequencing, and overexpression of the *Eco*RI endonuclease gene has been published recently (Wolfes et al., 1986). pRIF309+ contains the β-lactamase gene and the pMB1 replicon derived from pBR322, the *Eco*RI endonuclease gene under the control of the P_L promotor and the fd terminator, as well as the origin of replication of phage f1 (Figure 1). The P_L promotor in conjunction with the thermosensitive cI857 repressor allows the temperature-induced overexpression of the *Eco*RI endonuclease gene. Since expression of *Eco*RI endonuclease is lethal for cells lacking *Eco*RI methylase, it is necessary to supply an adequate amount of the cI repressor.

All cloning experiments were carried out in LK111(λ) ($lacI^q$, lac ZΔM15, lac Y^+) (Zabeau & Stanley, 1982), a kind gift of Dr. M. Zabeau (Gent), or WK6 mutS(λ) [Δ(lac-proAB), galE, strA, mutS215::Tn 10/F′, $lacI^q$, ZΔM 15, $proA^+B^+$], kindly supplied by M. Szardenings (Braunschweig).

*Eco*RI overexpression was performed in the cI857 repressor producing strain TGE900 [su^-, ilv^-, bio (ZcI857 ΔBamΔH1, F^-)], a kind gift of Dr. A. Ballard (Strasbourg). For this purpose TGE900 was transformed with pRIF309+ and pECOR4 (Bernard et al., 1979). The plasmid pECOR4 carries the chloramphenicol acetyltransferase gene, the p15A replicon derived from pACYC, and the *Eco*RI methylase gene. Induction of *Eco*RI endonuclease overproduction was initiated by a temperature shift from 32 to 42 °C.

Mutagenesis and sequencing was performed with the pRIF309+ single strand. Since pRIF309+ carries the f1 origin of replication, superinfection of F′ *Escherichia coli* strains carrying this plasmid with f1 phage leads to packaging and excretion of single-stranded plasmid DNA, similarly as described for the pEMBL (Dente et al., 1985) or pMAC (P.

Stanssens, C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H. J. Fritz, unpublished results) plasmids.

Production of Single-Stranded DNA from pRIF309+. Single-stranded DNA from pRIF309+ was produced from LK111(λ) carrying the plasmid by a procedure adapted from those developed for M13 (Hutchinson et al., 1978). The following method yields up to 10 μg of single-stranded DNA, but may be scaled up 10–100-fold. A 3-mL LB culture containing 100 μg/mL ampicillin is inoculated by a glycerol stock culture and incubated overnight at 37 °C; 400 μL of this culture is transferred to 20 mL of LB, containing 100 μg/mL ampicillin, kept for 3 h at 37 °C, at a turbidity of approximately 0.3 A^{600nm}, infected with 10^{11} pfu of f1 phage, and incubated for 2.5 h. After centrifugation at 24000*g* for 20 min PEG6000 and NaCl are added to the supernatant to give a 5% (w/v) PEG–0.6 M NaCl solution. After 15 min at room temperature and centrifugation at 8000*g* for 20 min the pellet is dissolved in 600 μL of 10 mM Tris·HCl, pH 8.0–1.0 mM EDTA. This solution is extracted several times with phenol until the aqueous phase is clear. The DNA is precipitated by addition of 100 μL of 5 M $NaClO_4$ and 500 μL of 2-propanol. After 10 min at −70 °C the DNA is collected by centrifugation and redissolved in 300 μL of 0.3 M sodium acetate, pH 4.8 and precipitated with 2 volumes of ethanol. After centrifugation the DNA is dried in a desiccator and dissolved in 50 μL of 10 mM Tris·HCl, pH 8.0–1 mM EDTA. This DNA preparation consists predominantly of single-stranded pRIF309+. The amount of f1 DNA can be kept below 25% by harvesting the cells in the early phase of infection.

Secondary Structure Predictions for Single-Stranded pRIF309+. We have used published nucleic acid secondary structure prediction algorithms (Zucker & Stiegler, 1981; Reckmann et al., 1985) to analyze the probability of occurrence of secondary structures in the single-stranded pRIF309+ DNA. As shown for the region coding for the sequence between Pro90 and Phe222, a stem–loop structure can be formed which might interfere with mutagenesis of Arg200 (vide infra).

Site-Directed Mutagenesis. The Arg200 → Lys mutant was accessible neither by the Zoller–Smith protocol (Zoller & Smith, 1982) which we had used for the Glu144 → Gln and Arg145 → Lys mutations nor by the Kunkel method (Kunkel, 1985) using dut⁻ung⁻ strains. The desired mutation, however, could be introduced with the Eckstein protocol (Taylor et al., 1985) and is described in detail elsewhere (Düsterhöft, 1987). Briefly, the mutation was introduced with the phosphorylated mismatch primer pTAGTCGATCTAAC*T*TATTTAATATACC (mismatch in italics) into the 185 base pair *Bgl*II/*Pst*I fragment of the *Eco*RI gene which had been cloned into the polylinker of M13mp18. The marker yield was 80%; the mutation was verified by sequencing. The mutated gene fragment was cut out by *Xho*II/*Pst*I digestion and cloned into the plasmid pHK514 which contains the *Eco*RI wild-type gene and which had been cleaved previously by *Bgl*II/*Pst*I digestion. The resulting plasmid pHK526 harboring the mutant *Eco*RI gene was transformed in the present study into LK111(λ). The 532 base pair *Hin*dIII/*Asu*II fragment was isolated from pHK526 by preparative electrophoresis in 1% (w/v) agarose, followed by electroelution, phenol/$CHCl_3$ extraction, and ethanol precipitation. For overexpression and further mutagenesis this fragment was exchanged for the corresponding fragment in pRIF309+. For this purpose pRIF309+ was digested with *Hin*dII and *Asu*II. The 4530 base pair fragment was isolated by preparative electrophoresis (vide supra). A total of 300 ng of this fragment was ligated overnight at 15 °C with 100 ng of the 532 base pair fragment in the presence of 1 unit of T4 DNA ligase in a volume of 20 μL. A total of 10 μL of the ligation mixture was each transformed into LK111(λ) and LK111(λ) containing the plasmid pECOR4. For this purpose the DNA sample was added to 100 μL of sterile 0.1 M $CaCl_2$ solution and mixed with 200 μL of competent cells which had been stored in 0.1 M $CaCl_2$–25% (v/v) glycerol at −70 °C and were thawed on ice. The transformation mixture was incubated for 45 min at 0 °C and then for 3 min at 42 °C and after addition of 700 μL of LB incubated for another 45 min at 37 °C before streaking out on LB agar plates containing 100 μg/mL ampicillin [for LK111(λ)] or 100 μg/mL ampicillin and 30 μg/mL chloramphenicol [for LK111(λ) containing pECOR4]. Transformation of LK111(λ) containing pECOR4 yielded 60 clones, nearly all of which contained the mutated gene, while only one clone was obtained with LK111(λ). We assume that the *Eco*RI gene on the open or closed circular plasmid DNA when introduced into a competent cell might be transiently expressed and thereby reduce the transformation efficiency. Transformation with supercoiled mutated pRIF309+ coding for the Lys200 mutant, however, and further propagation were possible in LK111(λ) in the absence of pECOR4, i.e., without *Eco*RI methylase being present. The same can be done with wild-type pRIF309+, indicating that repression of *Eco*RI gene expression is sufficiently strong in this system to allow for growth. The *Eco*RI gene of ten clones containing the insert was sequenced in the region of the mutation; five clones contained the desired mutation. The complete *Eco*RI gene of one of these clones was sequenced and found to contain only this mutation.

The double mutant Glu144Arg145 → GlnLys and the triple mutant Glu144Arg145Arg200 → GlnLysLys were produced by the gapped-duplex method (Kramer et al., 1981) starting from single-stranded pRIF309+ containing the wild-type gene or the mutated gene coding for the Lys200 mutant, respectively. The 4530 base pair *Hin*dIII/*Asu*II fragment of pRIF309+ (vide supra) was used as the gap-forming DNA. A total of 10 pmol of the phosphorylated mismatch primer pGGTAATGCTATCC*A*AA*A*ATCTCATAAGAATAT (mismatches in italics, the second mismatch destroys the single *Bgl*II site in pRIF309+) was mixed with 1 pmol of single-stranded wild-type pRIF309+ or mutated pRIF309+ which codes for the Lys200 mutant, 0.4 pmol of *Hin*dIII/*Asu*II fragment of pRIF309+, and 4 μL of 1.5 M KCl–0.1 M Tris·HCl, pH 7.5. The volume was adjusted to 40 μL with H_2O. This mixture was incubated at 100 °C for 10 min and then cooled down to room temperature over a period of 2 h. To a 16-μL aliquot containing approximately 400 ng of gapped duplex DNA, 8 μL of 625 mM KCl, 275 mM Tris·HCl, pH 7.5, 150 mM $MgCl_2$, 20 mM DTE, 500 μM ATP, 250 μM of each dNTP, 6 units of T4 DNA ligase, and 2 units of Klenow polymerase and 56 μL of H_2O were added. This mixture was kept at 0 °C for 10 min and then incubated at 25 °C for 1 h. The reaction was stopped by addition of 1 μL of 0.5 M EDTA/KOH, pH 8.0; the mixture was diluted 2-fold with water, extracted first with 200 μL of phenol/$CHCl_3$ (1:1 v/v) and then with 200 μL of $CHCl_3$/isoamyl alcohol (24:1 v/v), and then made 0.3 M in sodium acetate, pH 4.8. After precipitation of the DNA with 2 volumes of ethanol, the DNA was collected by centrifugation. The pellet was dried in a desiccator and dissolved in 25 μL of 10 mM Tris·HCl, pH 8.0–1 mM EDTA and stored at −20 °C. Transformation of competent WK6mutS(λ) cells with this DNA was carried out as described above for LK111(λ). A total of 200 μL of the transformation mixture was streaked out on LB agar plates

containing 100 μg/mL ampicillin. More than 100 clones were obtained after incubation at 37 °C overnight. The residual 800 μL was poured into 100 mL of AB3 medium (Difco) containing 100 μg/mL ampicillin and incubated overnight at 37 °C in a 500-mL Erlenmayer flask. Cells were harvested by centrifugation. Plasmid DNA was prepared from these cells by the alkaline lysis procedure (Birnboim & Doly, 1979). Approximately 100 μg of pRIF309+ mutant DNA was obtained, dissolved in 100 μL of 10 mM Tris·HCl, pH 8.0–1 mM EDTA, and stored at −20 °C. A total of 2 μL of the DNA preparation was used to transform competent LK111(λ) cells in a volume of 300 μL. Another 2-μL aliquot was incubated with 10 units of *Bgl*II for 1 h at 37 °C, heated for 10 min at 70 °C, cooled down, and then used for transformation of LK111(λ) cells. Both transformation mixtures were streaked out on LB agar plates containing 100 μg/mL ampicillin and incubated overnight at 37 °C. The untreated DNA yielded approximately 200 clones and the *Bgl*II-treated DNA approximately 100 clones. Representative clones were picked, isolated on LB agar plates containing 100 μg/mL ampicillin, and then used to inoculate 3-mL LB cultures supplemented with 100 μg/mL ampicillin. After incubation overnight at 37 °C cells were harvested and suspended in 200 μL of 50 mM Tris·HCl, pH 8.0–50 mM EDTA–15% (w/v) sucrose and 10 μL of lysozyme [12 mg/mL in 50% (v/v) glycerol]. This mixture was incubated for 20 min at 0 °C. After addition of 800 μL of H_2O the suspension was kept at 75 °C for 2 min and then extracted with 50 μL of phenol. After centrifugation at 40000*g* for 20 min the aqueous phase was collected and extracted with $CHCl_3$/isoamyl alcohol (24:1 v/v). DNA was precipitated with 100 μL of 5 M $NaClO_4$ and 500 μL of 2-propanol. The DNA was collected by centrifugation, dried in a desiccator, and dissolved in 50 μL of 1 mM Tris·HCl, pH 8.0–0.1 mM EDTA. A total of 10 mL of the DNA solution was incubated with 10 units of *Bgl*II for 60 min at 37 °C and analyzed on 1% (w/v) agarose gels. Plasmid DNA with the desired mutation lacks the single *Bgl*II site of wild-type pRIF309+; this allows rapid screening and selection of positive clones. The *Bgl*II digestion of the DNA prior to transformation increases the marker yield from 40% to 80%. The plasmid DNA of five positive clones was chosen for sequencing of the region of interest and found to have the desired mutations. The complete *Eco*RI gene was sequenced in one of these clones and found to have no other mutation.

Sequencing. All mutant genes were sequenced over the entire coding region by the dideoxy method (Sanger et al., 1977). For this purpose 5 μL containing 1 μg of single-stranded pRIF309+ DNA, 2 μL containing 5 ng of the appropriate primer, 2 μL of 0.1 M Tris·HCl, pH 7.4–0.1 M $MgCl_2$–0.5 M NaCl, and 8 μL of H_2O were mixed. The mixture was incubated at 95 °C for 5 min and allowed to cool down to room temperature. This solution was divided into four 4-μL portions, to which 2 μL of dNTP, 1.5 μL of ddNTP (N = A, C, G, or T), 1 μL corresponding to 1.5 μCi of [^{35}S]-α-thio-dATP (NEN), and 1 μL Klenow polymerase (1.25 units) were added. After incubation for 30 min at room temperature 2 μL of a dNTP chase solution was added and the incubation continued for 50 min. The reaction was stopped by addition of 40 μL of stop solution and 200 μL of ethanol and then left at −20 °C overnight. After centrifugation and removal of the supernatant the pellets were washed with 70% (v/v) ethanol and dissolved in 3 μL of formamide, 20 mM EDTA, and 0.1% (w/v) bromophenol blue. This mixture was incubated at 95 °C for 3 min and then put on ice. Electrophoresis was carried out on 6% (w/v) PAGE gels containing 8 M urea. The gels were exposed for 2 days at −80 °C with Kodak XAR5 films.

Transformation Assay in TGE900. Transformation of pRIF309+ into TGE900 which produces the thermosensitive cI857 repressor is necessary for overexpression of the *Eco*RI gene. Since overexpression of *Eco*RI activity is incompatible with growth in the absence of *Eco*RI methylase (O'Connor & Humphreys, 1982; Cheng & Modrich, 1983; Kuhn et al., 1986), transformation of supercoiled mutated pRIF309+ into TGE900 and TGE900 containing the plasmid pECOR4 which harbors the methylase gene allows the screening of mutants for endonucleolytic activity with *Eco*RI wild-type specificity. Only TGE900 (pECOR4) allows growth at 42 °C when an *Eco*RI mutant with wild-type specificity is expressed.

The transformation protocol was the same as described above, except that the heat shock temperature was reduced to 37 °C and the subsequent incubation was performed at 30 °C. Transformation mixtures were streaked out on LB agar plates containing either 100 μg/mL ampicillin or 100 μg/mL ampicillin and 30 μg/mL chloramphenicol, respectively. Half of the plates were incubated overnight at 28 °C and the other half at 42 °C. None of the mutants could be grown up in TGE900 at 42 °C. On the other hand, TGE900 containing pECOR4 could be grown at 42 °C in the presence of wild-type or mutant pRIF309+, demonstrating that all mutants had at least residual wild-type activity. Under noninducing conditions, i.e., at 28 °C, both TGE900 and TGE900 containing pECOR4 were viable when transformed with wild-type or mutant pRIF309+.

Fermentation of TGE900 Producing Mutant EcoRI. TGE900 clones carrying both pECOR4 and mutated pRIF309+ were grown up in 500-mL LB cultures at 32 °C overnight in the presence of 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. One 500-mL culture was used to inoculate two 8-L fermenters containing the same medium. Fermentation at 32 °C was carried out until a turbidity of $A^{600nm} = 0.5$ was reached, usually after 2 h. After a temperature shift to 42 °C fermentation was continued for another 3 h under pH control (pH 7.4). Cells were harvested by centrifugation at 4 °C, suspended in 10 mM Tris·HCl, pH 8.0, 0.1 mM EDTA, and 0.1 M NaCl buffer, and centrifuged again. The resulting pellet, usually 25 g per 8-L fermenter, was resuspended in 20 mL of buffer A [0.03 M potassium phosphate, pH 7.2, 1 mM EDTA, 0.1 mM 1,4-dithiothreitol, and 0.01% (w/v) Lubrol] containing 0.1 M NaCl (vide infra) and stored at −70 °C.

Purification of Mutant EcoRI Endonucleases. The purification of *Eco*RI mutants from an overproducing *E. coli* strain was carried out similarly as described recently (Wolfes et al., 1986). Approximately 100 g of wet cell paste, the typical yield of a 32-L fermentation, was suspended in 250 mL of buffer A, containing 0.1 M NaCl. Cells were broken up by sonication in a MSE MK2 sonifier at 150 W for 30 min with intermittent cooling. All subsequent steps were carried out at 4 °C. Cell debris was removed by centrifugation at 30000*g* for 30 min. The supernatant was adjusted to pH 7.2 with a concentrated NH_3 solution, diluted to give the conductivity of buffer A containing 0.1 M NaCl, and loaded onto a phosphocellulose (Whatman P-11) column (5 × 30 cm) equilibrated with buffer A containing 0.1 M NaCl. After the column was rinsed with 2 L of the same buffer, elution was carried out with a linear gradient of 2 × 3 L of 0.1–1.0 M NaCl in buffer A. Peak fractions containing the enzyme as assayed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) were pooled and concentrated after appropriate dilution with water on a phosphocellulose column (5 × 3 cm)

by elution with buffer A containing 1.0 M NaCl. The concentrated protein solution was diluted 5-fold with water and applied to a hydroxyapatite column (5 × 30 cm) equilibrated with buffer A containing 0.2 M NaCl. After the column was rinsed with 1 L of the same buffer, elution was carried out with 2 × 2.5 L of 0–0.47 M potassium phosphate in buffer A containing 0.2 M NaCl. Peak fractions containing the enzyme were pooled and concentrated on a small phosphocellulose column (1.5 × 3 cm) as described above. The concentrated enzyme solution was dialyzed against buffer A containing 0.3 M NaCl and 80% (v/v) glycerol and stored at −30 °C. This purification scheme gives within 5 days approximately 10 OD^{280} units of *Eco*RI which is at least 95% pure as judged by SDS–PAGE and isoelectric focusing (IEF). For the determination of the concentration of wild-type *Eco*RI and *Eco*RI mutants, we have used the extinction coefficient $E^{278nm} = 8.3$ as reported by Modrich and Zabel (1976) for wild-type *Eco*RI.

Isoelectric Focusing of EcoRI and EcoRI Mutants. IEF of *Eco*RI was carried out under native as well as under denaturing conditions. Native IEF was performed in 0.5 mm thick polyacrylamide gels [7.5% (w/v) acrylamide and 3% (w/v) bis(acrylamide)] containing 2.4% (w/v) ampholytes (Servalyt pH 3–10). The anolyte consisted of 25 mM L-aspartic acid and 25 mM glutamic acid; the catholyte was 2 M ethylenediamine, 25 mM L-arginine, and 25 mM L-lysine. Focusing was carried out on a Multiphor flat-bed electrophoresis apparatus (LKB) connected to a circulating cooling bath kept at 10 °C. Samples of approximately 20 μg of wild-type *Eco*RI or mutant were applied to 4 × 8 mm LKB filter paper strips which were placed at a distance of 1.5 or 4.0 cm from the anode electrophoresis wick. Focusing was begun at 500 V and after 30 min was continued at 2000 V for 2 h. Sperm whale myoglobin (p*I* = 8.3), horse myoglobin (p*I* = 7.3), and bovine carbonic anhydrase (p*I* = 5.9) were used as marker proteins. Protein bands were stained with 0.1% (w/v) Coomassie Blue R after fixation with 20% (w/v) trichloroacetic acid. IEF under denaturing conditions was carried out in the presence of urea. For this purpose solid urea, β-mercaptoethanol, and Servalyt (pH 3–10) were added to the sample to give a 9 M urea, 2% (v/v) β-mercaptoethanol, and 4% (w/v) ampholyte solution which was incubated overnight at 4 °C. Focusing was carried out as described above in polyacrylamide gels [4% (w/v) acrylamide, 3% (w/v) bis(acrylamide)] containing 8 M urea and 2.4% (w/v) Servalyt (pH 3–10).

*Analytical Gel Permeation Chromatography. Eco*RI mutants were analyzed by fast protein liquid chromatography on a Superose 12 HR 10/30 column (Pharmacia) installed into a Pharmacia FPLC system consisting of a LCC-500 controller, two P-500 pumps, a MV-7 motor valve, and a UV-M monitor. The resolution of this column is such that it gives a base-line separation of bovine serum albumin, M_r = 65K, β-lactoglobulin (goat), M_r = 37K, and cytochrome *c* (bovine heart), M_r = 13K, at a flow rate of 0.5 mL/min. Typically, 10 μL of a 10 μM *Eco*RI solution diluted to this concentration from the stock solution was injected into the valve and gel filtration carried out at a flow rate of 0.5 mL/min at ambient temperature. Four different buffers were used: (I) 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, 0.05 M NaCl; (II) 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, 0.8 M NaCl; (III) 0.02 M Tris·HCl, pH 7.2, 10 mM EDTA, 0.05 M NaCl; (IV) 0.02 M Tris·HCl, pH 7.2, 10 mM EDTA, 0.8 M NaCl. The chromatographic behavior of wild-type *Eco*RI was quite different in these buffer systems. While at low salt concentrations *Eco*RI was eluted after 17 mL, at high salt concentrations the predominant amount of *Eco*RI was eluted after 13.2 mL and only a small amount after 17 mL. Since bovine serum albumin and ovalbumin are eluted after 12 and 13.3 mL, respectively, it is assumed that under the conditions given and at high salt concentrations wild-type *Eco*RI is eluted mainly as a dimer, whereas at low salt concentration it is eluted as a monomer. Gel permeation chromatography on Superose 12 at high salt concentration, therefore, allows us to analyze whether mutations interfere with dimer formation.

Analytical Ultracentrifugation. Sedimentation velocity runs were carried out in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 800 mM NaCl at 22 °C in 12-mm cells in an An-G-Ti rotor at 48 000 rpm in a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control, a high-intensity ultraviolet illumination system, a photoelectric scanner, and an electronic multiplexer. Sedimentation profiles were recorded at 280 nm and stored in a digital recorder. The sedimentation of wild-type *Eco*RI and *Eco*RI mutants was analyzed in the same run. For this purpose the protein samples were dialyzed for 24 h at 4 °C against 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 800 mM NaCl. Immediately prior to the experiment, the protein samples were diluted with the dialysis buffer to approximately equal concentrations.

The sedimentation coefficients for *Eco*RI and *Eco*RI mutants were determined from the movement of the sedimenting boundary (Schachman, 1959). The shape and size of the protein molecules were estimated according to the method of Oncley (1941) derived for sedimenting hydrated particles.

Circular Dichroism. Circular dichroism spectra of wild-type and mutant *Eco*RI were recorded in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 0.8 M NaCl at ambient temperature in 0.05-cm cuvettes in a Jobin-Yvon Dichrograph R.J. Mark III calibrated with (+)-campher-10-sulfonic acid, D-pantolactone, and epiandrosterone. The bandwidth was 2 nm, the rate of scanning was 0.05 nm/s, and the time constant was 5 s. Spectra were stored in a digital recorder, processed, and analyzed numerically in terms of α-helix, β-pleated sheet, and residual structure content with myoglobin, lysozyme, lactate dehydrogenase, papain, and ribonuclease A as reference proteins (Chen et al., 1972).

Temperature-induced denaturation was monitored by circular dichroism spectroscopy. CD melting curves were recorded at 220 nm in 0.4-cm cuvettes with a temperature increase per time of 10 °C/h which was the same in all experiments to allow for a comparison of the thermal stability of wild-type *Eco*RI and *Eco*RI mutants.

Results

Mutagenesis. The site-directed mutagenesis protocol, a modified Zoller–Smith procedure with single-stranded plasmid DNA as the template and a nonadecamer mismatch primer, turned out to be unsuccessful in our hands to produce the Lys200 mutant. The reason for this most likely is that the codon for Arg200 is located in a region of extensive secondary structure (Figure 2). These difficulties could be overcome by using a larger mismatch primer (27mer) and an M13 template containing the relevant portion of the *Eco*RI gene and applying the Eckstein protocol with its inherently more favorable marker yield. This procedure resulted in approximately 80% positive clones.

The Gln144Lys145 double mutation and the Gln144Lys145Lys200 triple mutation were introduced by the gapped duplex method using the single-stranded wild-type pRIF309+ and the pRIF309+ template carrying the Lys200 mutation, respectively, and a 32mer double mismatch primer.

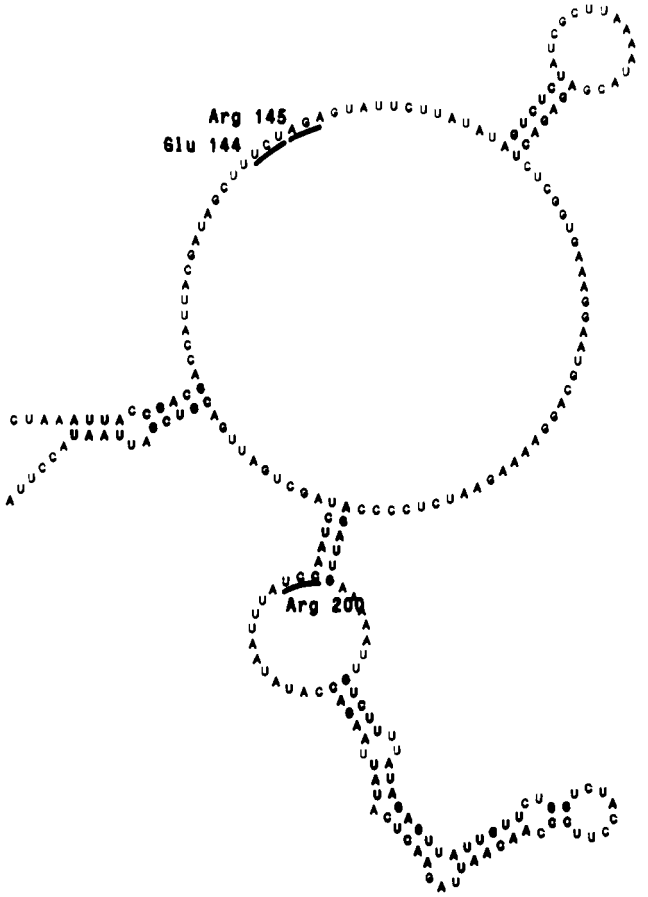

FIGURE 2: Secondary structure prediction for single-stranded pRIF309+. The secondary structure prediction program as developed for single-stranded RNA (this is the reason why U stands for T) was used to predict the secondary structure of single-stranded pRIF309+ DNA. Shown here is the portion of interest: while the codons for Glu144 and Arg145 are in an extended single-stranded region, the codon for Arg200 is part of a stem–loop structure which is calculated to be stable at 50 °C.

Transformation of the heteroduplex DNA into a repair-deficient *E. coli* strain resulted in approximately 40% positive clones, a yield which could be increased to approximately 80% by isolating the plasmid DNA, cleaving the wild-type DNA at a restriction site which was not present in the mutant DNA, and retransforming into *E. coli* cells. A flow sheet of the site-directed mutagenesis reaction is shown in Figure 3.

In Vivo Assay of the Activity of EcoRI Mutants. Since in higher concentrations the product of the *Eco*RI gene is lethal for the *E. coli* cell, care has to be taken that during the process of mutagenesis expression of the gene is efficiently repressed. This is achieved by keeping the gene under P_L control and working with λ lysogenic *E. coli* strains which produce sufficient cI repressor to block transcription of the *Eco*RI gene.

For the overexpression of *Eco*RI the plasmid harboring the *Eco*RI gene has to be transformed into *E. coli* cells that produce a thermosensitive cI repressor and sufficient *Eco*RI methylase to support cell growth during the time in which the toxic gene product, i.e., *Eco*RI endonuclease, accumulates. This is only possible for a limited period of time after induction, since the *Eco*RI endonuclease cleaves chromosomal DNA as well as the plasmid DNA, which carries the β-lactamase gene, also at noncanonical sites, albeit with very much reduced rate. The necessity to have the *Eco*RI methylase present for expression of the *Eco*RI endonuclease gene can be used for a sensitive screening of *Eco*RI mutants (Yanofsky et al., 1987): even if an *Eco*RI mutant has only residual endonucleolytic activity, overexpression is lethal for a cell in the absence of *Eco*RI methylase. By this criterion the Lys200 mutant, the Gln144Lys145 double mutant, and the Gln144Lys145Lys200 triple mutant have been shown to have *Eco*RI activity. This was confirmed with the purified proteins (see accompanying paper). The transformation assay also reveals that these mutants must have wild-type specificity, since the *Eco*RI methylase presumably is specific for canonical sites.

Table I: Isoelectric Points of Wild-Type *Eco*RI and *Eco*RI Mutants under Native and Denaturing Conditions

	native	denaturing
wild type	5.8 ± 0.05	7.1 ± 0.05
Lys200	5.7 ± 0.05	7.05 ± 0.05
Gln144Lys145	9.3 ± 0.05	8.7 ● 0.05
Gln144Lys145Lys200	9.3 ± 0.05	8.7 ± 0.05

Characterization of Mutant Enzymes. All *Eco*RI mutants were sequenced on the DNA level over the entire coding region to verify that only the desired mutation had been introduced. For the Gln144Lys145 double mutant and the Gln144Lys145Lys200 triplet mutant it was verified that the *E. coli* cells producing these mutant enzymes in the large-scale fermentations contained the mutant pRIF309+ which could be distinguished from wild-type pRIF309+ by the absence of the *Bgl*II site. The purified mutant proteins were characterized by SDS–PAGE (not shown) and demonstrated to have the same apparent subunit molecular weight as wild-type *Eco*RI. Their isoelectric point (p*I*) was determined by IEF, under both native and denaturing conditions (Table I). Under denaturing conditions wild-type *Eco*RI has an isoelectric point of 7.1, as expected from its content of 36 basic and 35 acidic amino acid residues (Newman et al., 1981; Greene et al., 1981). Under native conditions wild-type *Eco*RI is slightly more acidic.

The Arg200 → Lys mutant has the same p*I* value as wild-type *Eco*RI in both the absence and presence of urea. The double mutant Glu144Arg145 → GlnLys and the triple mutant Glu144Arg145Arg200 → GlnLysLys are more basic proteins than wild-type *Eco*RI or the Lys200 mutant. The p*I* difference of 1.6 pH units is large and indicates that the titration curve for *Eco*RI must have a steep rise around this pH. The difference under nondenaturing conditions is even larger, about 3.5 pH units, and can only be explained by major structural differences between these two mutants and wild-type *Eco*RI (vide infra).

Gel Filtration. Analytical gel permeation chromatography was carried out in order to find out whether the *Eco*RI mutants like wild-type *Eco*RI are dimers of identical subunits. We had previously performed these experiments in 0.03 M potassium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM DTE, 0.01% (w/v) Lubrol, and 0.2 M NaCl on AcA44 columns. The coelution of wild-type *Eco*RI and *Eco*RI mutants was taken as evidence for the unaffected dimerization potential of the mutant proteins (Wolfes et al., 1986). We have now carried out these experiments using fast protein liquid chromatography under the ionic conditions of the enzymatic assay, as well as with added extra salt. Wild-type *Eco*RI is eluted under assay conditions after ovalbumin (Figure 4a). At high salt concentrations, however, two peaks are observed: a dominant one slightly ahead of ovalbumin and a small one after ovalbumin (Figure 4b). In the absence of Mg^{2+} ions and in the presence of EDTA the same behavior is observed (data not shown). From the finding that the material eluting at high salt concentration at 13.2 mL upon rechromatography gives again rise to a dominant peak at 13.2 mL and a small peak at 17 mL, we conclude that the two species are in equilibrium with each other. The chromatographic behavior of *Eco*RI at low salt and at this

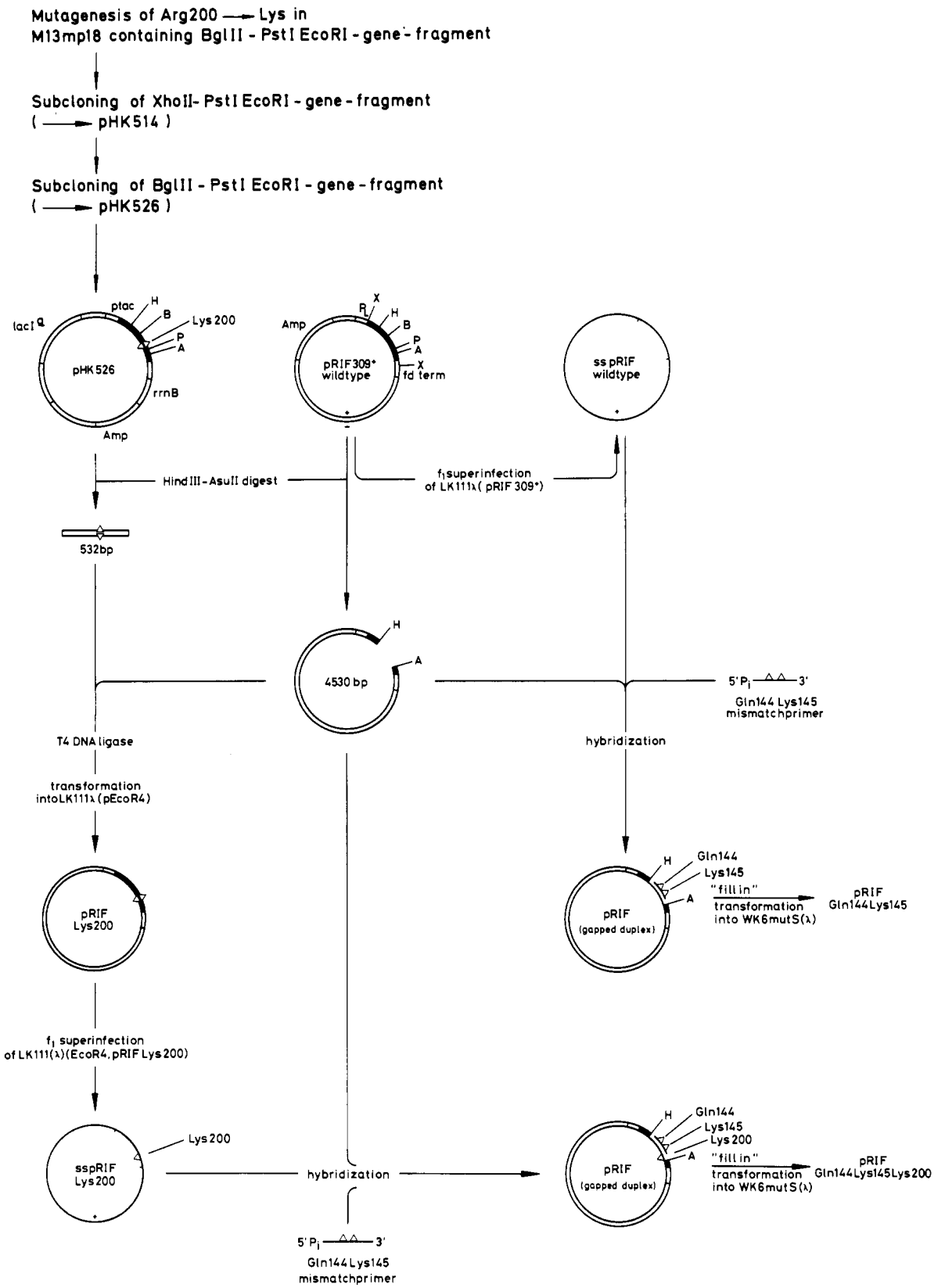

FIGURE 3: Flow sheet of the site-directed mutagenesis reactions.

protein concentration (~0.1 μM, due to dilution of the 10 μM sample during the gel filtration process) suggests that *Eco*RI is a monomer under these conditions and that in the presence of added salt the dimer is the predominant species. The dependence of the elution pattern on salt concentration indicates that above 0.2 M NaCl the dimer is the more stable species, while below 0.2 M NaCl the dimer is less stable than the monomer (Figure 4c). The relative large elution volume as compared to those of the globular reference proteins indicates that at low as well as high salt concentration *Eco*RI seems to interact with the Sepharose matrix, since a dimeric *Eco*RI should have an elution volume more similar to that of bovine serum albumin than to that of ovalbumin and a monomeric *Eco*RI should be eluted more closely to ovalbumin as observed. An interaction between *Eco*RI and the column matrix will give values for the relative molecular mass which are too small.

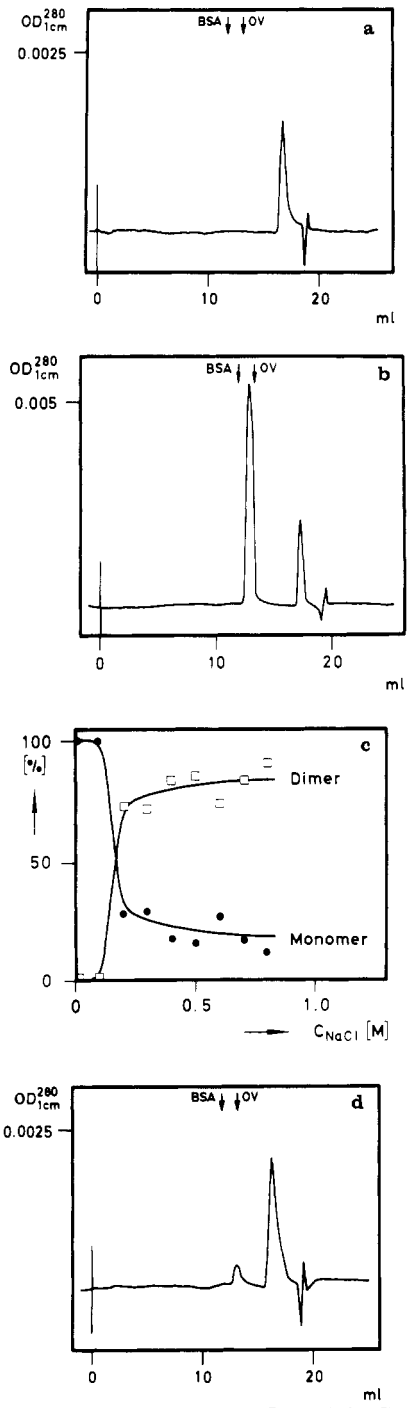

FIGURE 4: Gel filtration of wild-type *Eco*RI and the Gln144Lys145 double mutant. A total of 10 μL of a 15 μM solution of wild-type *Eco*RI in storage buffer was injected into the valve of the fast protein liquid chromatograph and chromatographed in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 0.05 M (a) or 0.8 M NaCl (b) on a gel filtration column calibrated with bovine serum albumin (M_r = 65000) and ovalbumin (M_r = 45000). Considerably less *Eco*RI is eluted from the gel filtration column in (a) as compared to that in (b), presumably because at low salt concentrations considerable amounts of *Eco*RI denature and precipitate out of solution. The ionic strength dependence of the monomer/dimer equilibrium is shown in (c). (d) is the elution profile obtained with 10 μL of a 45 μM solution of the Gln144Lys145 double mutant subjected to gel filtration in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 0.8 M NaCl.

Provided that this interaction is the same for *Eco*RI and the *Eco*RI mutants studied here, one can obtain information on the quaternary structure of wild-type *Eco*RI and *Eco*RI mutants. The important point for the present study is that the Lys200 mutant behaves like wild-type *Eco*RI (data not shown),

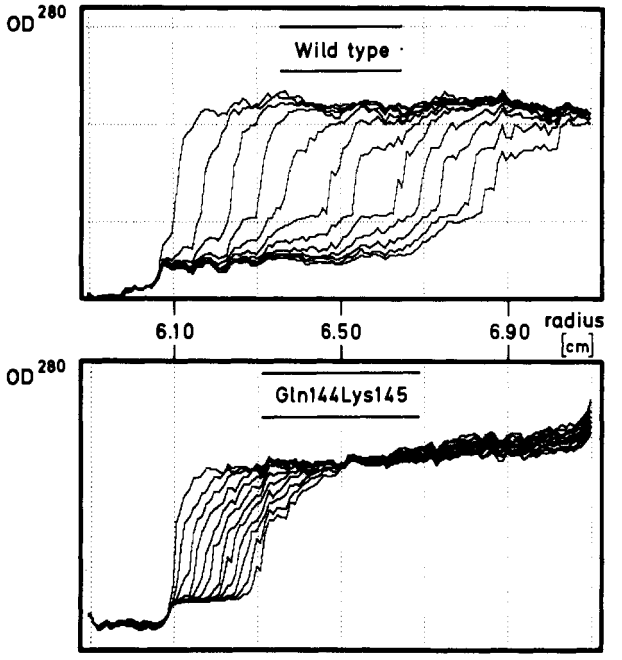

FIGURE 5: Sedimentation velocity runs with wild-type and mutant *Eco*RI preparations in the analytical ultracentrifuge. Wild-type (top) and mutant (Gln144Lys145) *Eco*RI were sedimented in the presence of 0.8 M NaCl. Protein concentration profiles were obtained in approximately 10-min intervals and evaluated for the position of the boundary. From a half-logarithmic plot (ln boundary position vs time) the s values as given in Table II were evaluated.

Table II: Sedimentation Coefficients of Wild-Type *Eco*RI and *Eco*RI Mutants at High Ionic Strength

	s^{app} (S)
wild type	6.8 ± 0.2
Lys200	6.0 ± 0.2
Gln144Lys145	1.8 ± 0.2
Gln144Lys145Lys200	1.6 ± 0.2

while the double mutant Gln144Lys145 (Figure 4d) and the triple mutant Gln144Lys145Lys200 (data not shown) have a much higher propensity to be monomers at high salt concentration than wild-type *Eco*RI or the Lys200 mutant.

Analytical Ultracentrifugation. The difference in the quaternary structure between wild-type *Eco*RI and the Lys200 mutant on one had and the double and triple mutants on the other hand is also evident in a sedimentation analysis. These experiments were carried out only at high salt concentrations. Figure 5 shows the pronounced difference in the sedimentation behavior of wild-type *Eco*RI and the double mutant Gln144Lys145. While the wild-type enzyme sediments with a sedimentation coefficient of 6.8 S, the double mutant is characterized by an s value of 1.8 S. There is no indication of an increase in sedimentation coefficient with time, which one would expect if the dimer/tetramer equilibrium was sensitive to pressure. As is obvious from Table II the Lys200 mutant behaves like the wild-type enzyme and the triple mutant like the double mutant (Table II). Different from the gel filtration experiments described above, sedimentation velocity runs in the analytical ultracentrifuge allow experiments to be carried out at accurately known protein concentrations in homogeneous solution. The sedimentation velocity runs were carried out at approximately 3.5–4.5 μM concentrations (based on the molecular weight of the dimer). At these concentrations *Eco*RI has been reported to be a tetramer (Modrich & Zabel, 1976). Indeed, an s value of 6.8 or 6.0 S is not compatible with an *Eco*RI dimer but rather indicates that it is a tetramer of somewhat elongated shape under these conditions or that

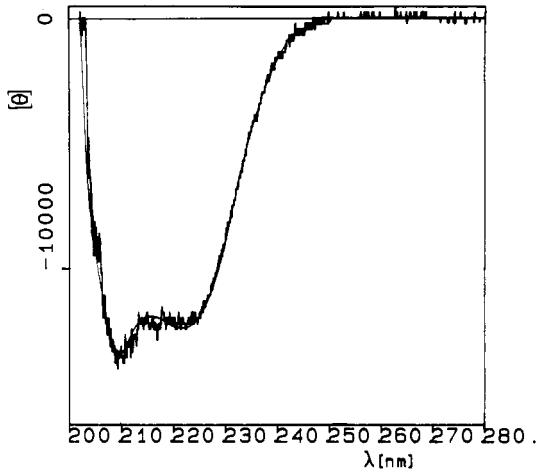

FIGURE 6: Circular dichroism spectrum of the Gln144Lys145 double mutant. The experimental CD spectrum of 0.81 mg/mL Gln144Lys145 double mutant in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 0.8 M NaCl is shown. Superimposed is the theoretical spectrum for a protein of 41% α-helix and 16% β-pleated sheet.

dimers and tetramers of globular shape are in equilibrium with each other. The double and triple mutants with *s* values of 1.8 and 1.6 S are definitely neither tetramers nor dimers under these conditions, but presumably partially unfolded and, therefore, highly hydrated monomers. Similar experiments could not be carried out under assay conditions, i.e., at low salt concentration, since during sample preparation which involves a 24-h dialysis against the assay buffer both wild-type *Eco*RI and the *Eco*RI mutants precipitated. This problem probably also occurred in the gel filtration experiments, since at low salt concentration a considerable amount of the protein was not eluted from the column (see legend to Figure 4). We assume that in the absence of substrate and of stabilizing detergents and at low salt concentration tetrameric or dimeric *Eco*RI dissociates to the monomer with partially unfolds and then eventually forms aggregates which precipitate out of the solution. Since the gel filtration and the sedimentation experiments were carried out at different concentrations, they are not comparable in a strict sense. But they are still comparable in the sense that at the same salt concentration changes in protein concentration should shift the monomer–dimer–tetramer equilibria. The analytical ultracentrifuge runs confirm, therefore, the result of the gel filtration experiments that the simultaneous replacement of Glu144 by Gln *and Arg145* by Lys interferes with the formation of the quaternary structure characteristic for wild-type *Eco*RI. This result may also explain the aberrant p*I* values of the double and triple mutants.

Circular Dichroism. Circular dichroism (CD) spectra of wild-type *Eco*RI and the various *Eco*RI mutants were recorded in order to find out whether the mutations introduced into *Eco*RI affected the secondary structure of this protein. The Cotton effects due to the peptide bond absorption are sensitive to small changes in the content of α-helix structure and to a smaller degree also to differences in the content of β-pleated sheet and residual structures. If the CD spectrum of an *Eco*RI mutant is unchanged with respect to that of the wild type, this can be taken as evidence that the secondary structure composition is the same; if it is different, the difference can be analyzed in quantitative terms. Figure 6 shows the CD spectrum of the Gln144Lys145 mutant recorded at high salt concentration to allow for a comparison with the results of the gel filtration experiments and analytical ultracentrifuge runs. In Table III the secondary structure analysis based on these

Table III: Secondary Structure Analysis of Wild-Type *Eco*RI and *Eco*RI Mutants at High Ionic Strength

	α-helix (%)	β-pleated sheet (%)
wild type	40 ± 5	19 ± 3
Lys200	39 ± 5	12 ± 3
Gln144Lys145	41 ± 5	16 ± 3
Gln144Lys145Lys200	37 ± 5	17 ± 3

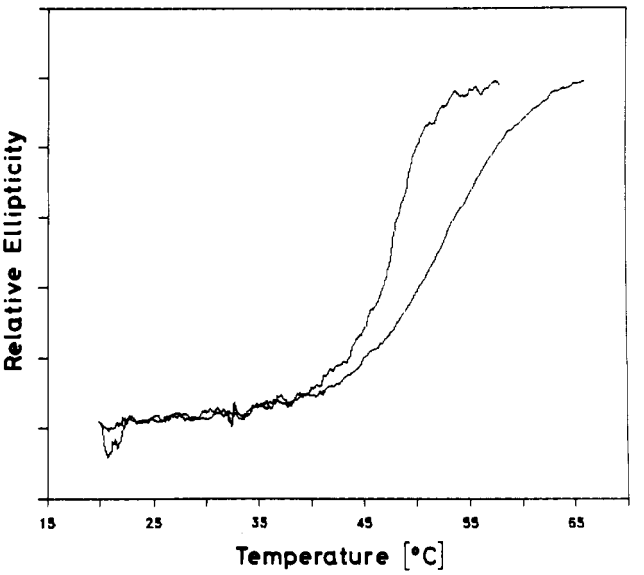

FIGURE 7: Temperature-induced denaturation of wild-type *Eco*RI and the Gln144Lys145 double mutant. The temperature-induced denaturation of 0.066 mg/mL wild-type *Eco*RI (upper curve, T_M = 47.6 °C) and of the Gln144Lys145 double mutant (lower curve, T_M = 52.2 °C) in 0.02 M Tris·HCl, pH 7.2, 10 mM $MgCl_2$, and 0.8 M NaCl was recorded by measuring the reduction in negative ellipticity at 220 nm (cf. Figure 6). Denaturation of wild-type *Eco*RI in contrast to the Gln144Lys145 double mutant was accompanied by aggregation and was not reversed by returning to starting conditions.

spectra is given. It is clear from these results that the double and triple mutants have a secondary structure very similar to that of the wild-type enzyme, in spite of the fact that they are affected in their ability to form a dimer. It is noteworthy that the secondary structure composition of wild-type *Eco*RI and the *Eco*RI mutants measured at high salt concentration (37–41% α-helix, 12–19% β-pleated sheet) is slightly different from that determined at lower salt concentrations (33–36% α-helix, 21–25% β-pleated sheet) [data for the wild type reported by Goppelt et al. (1980) and Manavalan et al. (1984); data for the mutants not shown].

In order to find out whether the diminished dimerization potential affects the stability of the protein toward temperature-induced denaturation, we have analyzed the "melting" of the secondary structure of wild-type *Eco*RI, the Lys200 mutant, and Gln144Lys145 double mutant, and the Gln144Lys145Lys200 triple mutant. As can be seen in Figure 7 wild-type *Eco*RI shows a monophasic denaturation profile with a midpoint ("T_M") of 47.6 °C. The Gln144Lys145 double mutant displays a broader transition curve with a T_M of 52.2 °C. Most significantly, while the denaturation process is irreversible with wild-type *Eco*RI, it is fully reversible with the Gln144Lys145 double mutant. Thus, the conservative replacement of two amino acid residues located at the end of an α-helix which is part of the DNA binding site affects not only the monomer/dimer equilibrium but also the course of the thermal denaturation process. Contrary to our experiments, the predominantly monomeric Gln144Lys145 double mutant is slightly more stable than the dimeric wild-type enzyme and can be reversibly denatured by heating to 60 °C,

while wild-type *Eco*RI aggregates in an irreversible manner under the same conditions. The Gln144Lys145Lys200 triple mutant shows a reversible melting behavior very similar to that of the Gln144Lys145 double mutant, with a T_M of 50.9 °C, while the Lys200 mutant, like wild-type *Eco*RI, is denatured in an irreversible manner (not shown).

Discussion

The aim of the present study was to produce genetically engineered *Eco*RI mutants with an altered hydrogen-bonding potential in the DNA binding site. According to the X-ray structural analysis of an *Eco*RI–oligonucleotide complex, three amino acid residues are involved in forming specific contacts via hydrogen bonds to the substrate: Glu144, Arg145, and Arg200 (McClarin et al., 1986). In addition, these amino acid residues interact with other amino acid residues, thereby stabilizing the native conformation of the polypeptide as well as the dimeric structure of the protein. Glu144 has been reported to be involved in electrostatic contacts with Arg200 and Arg203 and to be in close contact with Arg145 of the other subunit across the subunit interface (Yanofsky et al., 1987). In agreement with this assignment is the finding that the Glu144 → Lys mutant obtained by random hydroxylamine mutagenesis is a monomer, as determined by gel filtration experiments as similarly described here. This mutant has no enzymatic activity, presumably also because of its inability to form a functional dimer under assay conditions (Yanofsky et al., 1987). The conservative Glu144 → Gln mutant and the Arg145 → Lys mutant, however, are dimeric proteins with reduced activity (Wolfes et al., 1986).

The potential of *Eco*RI to form a dimer cannot be determined under the buffer conditions of the enzymatic assay, since *Eco*RI in the absence of its substrate is unstable at low salt concentrations. At a salt concentration above 0.2 M, wild-type *Eco*RI forms a dimer or tetramer, depending on the protein concentration. It is demonstrated in the present paper that the Lys200 mutant is a dimer under conditions where the wild-type *Eco*RI is also dimeric. Since the substitution of Arg145 and Arg200 by lysine leaves the electrostatic interactions essentially intact, dimer formation is not impaired. In the Glu144 → Gln mutant these interactions are abolished, but since dimer formation is still possible, it may be argued that dipolar interactions can partially substitute for the stronger electrostatic interactions. The simultaneous substitution of Arg145 by lysine and Glu144 by glutamine, however, impairs but does not completely prevent the dimerization process. Since a lysine side chain is by approximately 0.1 nm shorter than an arginine side chain and has also a different charge distribution, it could be that the dipolar interactions between the two subunits of the double mutant are too weak to support the dimeric structure. The triple mutant is with respect to its structural properties very similar to the double mutant. Circular dichroism spectroscopy demonstrates that the preference of the double and triple mutant for the monomeric state is not accompanied by a significantly different secondary structure as compared to that of wild-type *Eco*RI. Our finding that dimerization is impaired but not completely abolished with the double and triple mutants may explain their aberrant behavior in isoelectric focusing gels and, more importantly, why these mutants in contrast to the Glu144 → Lys mutant (Yanofsky et al., 1987) retain some enzymatic activity. Different from wild-type *Eco*RI which is denatured irreversibly by heating to 60 °C, the Gln144Lys145 double mutant and the Gln144Lys145Lys200 triple mutant can be unfolded in a fully reversible manner as shown by circular dichroism spectroscopy.

Our gel filtration results suggest that at low salt concentration wild-type *Eco*RI at a protein concentration of ≤0.1 μM is predominantly in a monomeric form. At salt concentrations above 0.2 M and presumably also in the presence of the charged nucleic acid substrate the equilibrium is shifted toward the dimeric form which is catalytically active (Alves et al., 1982). The ultracentrifugation experiments show that at protein concentrations above 1 μM *Eco*RI forms tetramers, which have been shown previously to be enzymatically active, too (Langowski et al., 1981). A disturbance of these equilibria will affect the enzymatic activity and in an extreme case produce a totally inactive mutant, as shown previously (Yanofsky et al., 1987). On the other hand, if mutants, which have been shown at high salt concentration (the necessary prerequisite to carry out this investigation) to prefer the monomeric state, are less active than wild-type *Eco*RI, then part of this deficiency can be attributed to a disturbance of the monomer/dimer equilibrium (see accompanying paper).

The central conclusion of the accompanying paper is that the *Eco*RI mutants whose genetic engineering and characterization are described here and which have a drastically altered hydrogen-bonding potential toward their DNA substrate have reduced enzymatic activity but apparently have specificity similar to that of the wild-type enzyme. In view of the importance of this result and the controversy it presumably will generate, we have taken care to exclude all plausible artifacts:

(I) A single clone whose *Eco*RI gene was sequenced entirely was used for the large-scale fermentation of each mutant.

(II) After the fermentation the plasmid DNA contained in the *E. coli* cells which were broken up for the isolation of the *Eco*RI mutants was checked for carrying the mutant *Eco*RI gene.

(III) For the isolation of *Eco*RI mutants only fresh or HCl/NaOH-treated chromatography media were used. All glassware had been cleaned with utmost care and sterilized at 150 °C.

These precautions exclude contamination with wild-type *Eco*RI and other enzymes during the preparation; they cannot rule out the possibility of partial reversion of the mutated *Eco*RI gene during the fermentation which, however, is unlikely considering the absence of a selective pressure for reversion. Our physical data can exclude revertants in a concentration of >1%. Since we have measured specific activities for the *Eco*RI mutants which are by 2–4 orders of magnitude smaller than that of wild-type *Eco*RI, these measurements cannot per se exclude contaminations below this level. However, the different behavior of the *Eco*RI mutants as compared to wild-type *Eco*RI toward different substrates, viz., bacteriophage λ DNA, plasmid pUC8 DNA, and synthetic oligodeoxynucleotide (see accompanying paper), strongly argues against any contamination.

Acknowledgments

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Registry No. L-Glu, 56-86-0; L-Arg, 74-79-3; L-Gln, 56-85-9; L-Lys, 56-87-1; *Eco*RI endonuclease, 80498-17-5.

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