Biochemistry (Moscow), Vol. 65, No. 9, 2000, pp. 1006-1010. Translated from Biokhimiya, Vol. 65, No. 9, 2000, pp. 1190-1195.
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ACCELERATED PUBLICATION

A Study of the Asp110–Glu112 Region of *Eco*RII Restriction Endonuclease by Site-Directed Mutagenesis

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Received November 18, 1999
Revision received June 19, 2000

Abstract—Site-directed mutagenesis of the *ecoRII* gene has been used to search for the active site of the *Eco*RII restriction endonuclease. Plasmids with point mutations in *ecoRII* gene resulting in substitutions of amino acid residues in the Asp110–Glu112 region of the *Eco*RII endonuclease (Asp110 → Lys, Asn, Thr, Val, or Ile; Pro111 → Arg, His, Ala, or Leu; Glu112 → Lys, Gln, or Asp) have been constructed. When expressed in *E. coli*, all these plasmids displayed *Eco*RII endonuclease activity. We also constructed a plasmid containing a mutant *ecoRII* gene with deletion of the sequence coding the Gln109–Pro111 region of the protein. This mutant protein had no *Eco*RII endonuclease activity. The data suggest that Asp110, Pro111, and Glu112 residues do not participate in the formation of the *Eco*RII active site. However, this region seems to be relevant for the formation of the tertiary structure of the *Eco*RII endonuclease.

Key words: site-directed mutagenesis, *ecoRII* gene, *Eco*RII restriction endonuclease mutants

Type II restriction endonucleases are enzymes that interact specifically with DNA; in the presence of Mg^{2+}, they cleave the DNA in certain sites. More than 2900 such enzymes are known at present [1], many of them being widely used in genetic engineering. However, the mechanism of their interaction with substrate DNAs remains insufficiently studied. Amino acid sequences of many restriction enzymes have been determined; as a rule, the enzymes display no marked homology in their primary structures [2, 3]. Data on the crystal structures of five type II restriction endonucleases (*Eco*RI, *Eco*RV, *Bam*HI, *Pvu*II, and *Cfr*10I) as well as their DNA complexes are now available [2, 3]. From X-ray structure analysis, the amino acids participating in the formation of the active sites of these enzymes have been determined, and a similarity in their active site structures is revealed. A basic motif for the amino acid sequences in the active sites was proposed: P (E/D) X_n (E/D) Z K. This motif is not completely conservative. For instance, the proline residue may be absent in some restriction endonuclease active sites [2-4]. Also, it is known that both the *Fok*I (a type IIs restriction endonuclease whose crystal structure was recently determined) [5, 6] and the phage λ exonuclease [7] active sites resemble the active sites of the five above-mentioned restriction endonucleases. These data suggest structural similarity of restriction endonuclease active sites [2-4, 8].

*Eco*RII restriction endonuclease belongs to the type IIe restriction enzymes, which need the recognition of two DNA sites for their catalytic act [9]. The active enzyme–substrate complex consists of two identical subunits of *Eco*RII endonuclease interacting with two specific DNA sites [10]. But the crystal structure of *Eco*RII restriction endonuclease as well as of other IIe type enzymes has not been determined. Homology exists between the C-terminal amino acid sequence of *Eco*RII endonuclease and motifs characterizing the integrase family of recombinases [11]. Recently, the sites responsible for specific DNA-binding were determined in *Eco*RII endonuclease [12]. Of special interest is the determination of amino acid residues involved in the active site of *Eco*RII endonuclease. Site-directed mutagenesis is one of the most effective approaches in such investigations. In the present study, we attempted to use this method for evaluation of the involvement of distinct amino acid residues in formation of the active site of *Eco*RII restriction endonuclease. To do this, we selected a region in the *Eco*RII endonuclease molecule for "protein design", performed site-directed mutagenesis to produce a number of mutant *ecoRII* genes, expressed these genes, and then tested the gene products to determine whether they possessed *Eco*RII endonuclease activity.

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MATERIALS AND METHODS

The enzymes T4 polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, *E. coli* DNA polymerase I, exonuclease III, and *Bam*HI and *Hind*III restriction endonucleases were purchased from MBI Fermentas (Lithuania), *Eco*RII restriction endonuclease was provided by N. I. Matvienko (Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia).

The JM109 strain of *E. coli* (Stratagene, USA) was used for gene engineering work. The pR224 plasmid containing the *ecoRII* gene was kindly provided by Professor A. Bhagwat (Wayne State University, Detroit, USA).

Oligodeoxyribonucleotides were synthesized using a standard automated solid-phase amidophosphite method. We used the individual oligodeoxyribonucleotides:

d(CCCCCTTGCTCATCA) (I)

and

d(pCTGACGCTCCTGGCTTTCA) (II)

as well as mixtures of compounds of similar structure:

d(pGCCCACTTCAG**VHW**CCTGAAAATACAGGGGCT) (III),

d(pCCACTTCAGGAT**SN**TGAAAATACAGGGGCT) (IV),

and

d(pCTTCAGGATCCT**VHW**AATACAGGGGCT) (V),

where **N** = A, C, G, or T; **V** = A, C, or G; **H** = A, C, or T; **W** = A or T; **S** = C or G.

The mutagenic oligodeoxyribonucleotides III-V were synthesized using balanced mixtures of nucleotide synthons for appropriate positions [13].

Mutagenesis. The pR224 plasmid has two *Hind*III restriction sites. To remove one that lies beyond the boundaries of the *ecoRII* gene, we treated the pR224 plasmid with *Hind*III endonuclease (18°C, 2 min), isolated the linearized plasmid (which was cleaved at only one of two *Hind*III restriction sites) by agarose gel electrophoresis, processed the cohesive ends of the plasmid with T4 DNA polymerase, and ligated them with DNA ligase. The resulting product was used for transformation of *E. coli* cells, and cloned plasmids were selected by restriction analysis. The pR224H plasmid contained a single *Hind*III restriction site inside the *ecoRII* gene. The nucleotide sequence of the *ecoRII* gene matched the previously reported sequence [14] with the exception of the region encoding amino acid residues 103-113, this being in agreement with [15].

Site-directed mutagenesis of the "Asp110" and "Pro111" codons of the *ecoRII* gene was performed using the pR224H plasmid. To facilitate initial selection of mutants, site-directed mutagenesis of the "Glu112" codon of the *ecoRII* gene was performed using the previously constructed mutant plasmid pR224HB ("Pro111" → "Ala111"), which had no *Bam*HI restriction site.

Nucleotide substitutions in the *ecoRII* gene were performed as described in [16] using plasmids purified by centrifugation in a CsCl density gradient [17]. We cleaved plasmids pR224H or pR224HB with *Hind*III restriction endonuclease, partially hydrolyzed them with exonuclease III to obtain single-stranded ends of length 100-200 nucleotides, hybridized with both 15-20% molar excess of the mutagen oligodeoxyribonucleotide (III, IV, or V) and a pair of adapter oligodeoxyribonucleotides (I and II), then processed with DNA ligase to obtain a circular form, built-up the single-stranded sequences with T4 DNA polymerase, and processed again with DNA ligase. The initial stages of DNA restructuring, namely, the cleavage at the *Hind*III site and the partial hydrolysis with exonuclease III, were performed with monitoring using 0.6% agarose gel electrophoresis. The resulting heteroduplex was sedimented with ethanol, treated with DNA polymerase I, and then used for the transformation of *E. coli* cells. We examined the existence of *Hind*III and *Bam*HI restriction sites in cloned plasmids. The *Hind*III restriction site should persist in all "target" plasmid mutations, that is, in which the nucleotide sequence of the mutating region corresponds to the sequence of the mutagenic oligodeoxyribonucleotides used. The *Bam*HI restriction site should disappear in mutants in both the "Asp110" and "Pro111" codons, whereas it should be restored in mutants in the "Glu112" codon. The nucleotide sequence of the region subjected to site-directed mutagenesis was examined in previously selected plasmids using Sanger's sequencing method.

To obtain a deletion mutant, the pR224H plasmid was cleaved at the unique *Bam*HI restriction site, the linearized plasmid was treated with nuclease S1 for removing the single-stranded and small double-stranded terminal sequences, and then ligated with DNA ligase. After the transformation of *E. coli* cells, the clones expressing the mutant *Eco*RII restriction endonuclease forms were screened using a direct electrophoretic assay (10% SDS-PAGE) of cell-extract proteins. The nucleotide sequence of the deletion region of the *ecoRII* gene was determined in plasmids extracted from the cells expressing *Eco*RII proteins using Sanger's sequencing method.

All gene engineering work was performed using standard protocols [17].

Revealing of the mutant forms of *Eco*RII enzyme and determination of *Eco*RII endonuclease activity. Cell extracts were prepared as described previously [14] with slight modifications. *E. coli* cells from 0.5 ml of overnight

culture were harvested by centrifugation, suspended in 50 μl of 0.05 M sodium phosphate, pH 8.8, containing 0.3 M NaCl and lysozyme (4 mg/ml), incubated 15 min on ice, and disrupted by two freeze–thaw treatments. The resulting suspension was centrifuged (15 min, 12,000*g*, 4°C), and the supernatant ("cell extract") was mixed with two volumes of ice-cold buffer for storage of the *Eco*RII endonuclease (0.2 M Tris-HCl, pH 7.5, 0.25 M NaCl, 0.025 M $MgCl_2$, 0.035 M dithiothreitol, 50% glycerol) and used directly for the measurement of *Eco*RII endonuclease activity (see below). Pellets were washed with 0.2 ml of 0.05 M sodium phosphate buffer (pH 8.8) containing 0.3 M NaCl and Triton X-100 (1%), then washed with the same buffer without Triton X-100, dissolved in 60 μl of 0.062 M Tris-HCl (pH 6.8) containing 2-mercaptoethanol (5%), SDS (2%), and urea (8 M). The solution was heated for 5 min at 90°C, and the proteins were assayed by electrophoresis in 10% polyacrylamide gel with SDS.

For determination of *Eco*RII endonuclease activity, non-methylated pBR322 plasmid (0.3 μg) was added to the cell extracts (2 μl) in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.01 M $MgCl_2$ (10 μl total volume). The mixture was incubated for 40 min at 37°C and examined by electrophoresis in 1.4% agarose gel.

All tests and assays were repeated at last twice for all plasmid constructions described, producing identical results.

RESULTS AND DISCUSSION

We chosen a region of the *Eco*RII restriction endonuclease for mutagenesis taking into consideration the crystal structure of *Eco*RI, *Eco*RV, *Bam*HI, *Pvu*II, and *Cfr*10I restriction endonucleases as well as the general structure of their active sites—the P (E/D) X_n (E/D) Z K motif [2-4]. The *Eco*RII restriction endonuclease has at least three such motifs: P76 D77 X_{18} E96 K97, D110 P111 E113 X_{17} D130 C131 K132, and P314 V315 E316 X_{22} D339 K340. The region D110–P111–E112 in motif D110 P111 E113 X_{17} D130 C131 K132 was chosen as the first to be subjected to mutation analysis. We planned to substitute the amino acid residues in each position specified. The substitution of Asp110, Pro111, and/or Glu112 should result in complete loss of enzymatic activity if each of these residues or at least one of them is involved in formation of the *Eco*RII restriction endonuclease active site, as has been demonstrated for the substitution of Asp74 or Asp90 in the *Eco*RV endonuclease molecule [18].

Oligodeoxyribonucleotides were designed for the site-directed mutagenesis so that we could obtain a full set of planned substitutions in the individual codon for the chosen amino acid residue after a single round of mutagenesis. Three mixtures of mutagenic oligodeoxyribonucleotides were used (III, IV, or V, see "Materials and Methods"), each resulting in the *ecoRII* gene mutation set characterized by substitution of Asp110, Pro111, or Glu112, respectively, in the protein molecule.

Site-directed mutagenesis was performed in the following region of the *ecoRII* gene (the nucleotide substitution area is marked by the bold symbols):

```
             110   112
          GlnAspProGluAsn
...CCCACTTCAGGATCCTGAAAATACAGGGGCTCTGACGCTCCTGGCTTTCAAGCTTG...
              BamHI                                   HindIII
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Initial selection of target mutant clones was performed using plasmid hydrolysis with both *Hind*III and *Bam*HI endonucleases. Restriction analysis displayed a mutagenesis pattern characteristic for the method used [16]. A high yield (>96%) of plasmids with restored *Hind*III site was achieved in all experiments, indicating accurate following of the steps of the initial plasmid treatment protocol. Most of the plasmids analyzed after mutagenesis (70-76%) were identical to the initial plasmid. Approximately 21-24% of the plasmids (target mutants) demonstrated some modifications in the region of the *Bam*HI site. Three to six percent of the plasmids (side mutations, mainly deletions) demonstrated lack of the *Hind*III site and alterations (in some instances significant decrease) in their molecular masses. The average yield of target mutants was calculated as the ratio of the number of *Bam*HI endonuclease-resistant clones (disappearance of the *Bam*HI site in the case of mutagenesis in the region of the "Asp110" or "Pro111" codons) or *Bam*HI endonuclease-sensitive clones (restoration of *Bam*HI site in the case of mutagenesis in the region of the "Glu112" codon) to the total number of clones analyzed. Yields of target mutants were 22% (12 out of 54 clones) for mutagenesis in the region of the "Asp110" codon, 24% (10 out of 42 clones) for mutagenesis in the region of the "Pro111" codon, and 21% (10 out of 48 clones) for mutagenesis in the region of the "Glu112" codon. Sequencing revealed the following codon substitutions: GAT (Asp110) → AAA, AAT, ACT, GTA, or ATT; CCT (Pro111) → CGT, CAT, GCT, or CTT; GAA (Glu112) → AAA, CAA, or GAT (see table).

Aside from above-listed plasmids, which have codon substitutions, the plasmid carrying deletion of the sequence CAGGATCCT encoding amino acids Gln109–Pro111 in the *ecoRII* gene (table) was designed using standard gene engineering methods (plasmid cleavage at the unique *Bam*HI site, hydrolysis with S1 nuclease, ligation) with the screening of clones expressing the "full-size" *ecoRII* gene product.

Direct assay of cell proteins in SDS polyacrylamide gels by the method of Laemmli indicated that *E. coli* JM109 strains carrying all modified plasmids produce

Mutant *Eco*RII proteins with substituted or deleted amino acid residues

No.	Initial amino acid sequence	Mutation type	Resulting amino acid sequence	*Eco*RII endonuclease activity
1	-Gln-**Asp**(110)-Pro-	substitution	-Gln-**Lys**(110)-Pro-	yes
2	— " —	— " —	-Gln-**Asn**(110)-Pro-	— " —
3	— " —	— " —	-Gln-**Thr**(110)-Pro-	— " —
4	— " —	— " —	-Gln-**Val**(110)-Pro-	— " —
5	— " —	— " —	-Gln-**Ile**(110)-Pro-	— " —
6	-Asp-**Pro**(111)-Glu-	— " —	-Asp-**Arg**(111)-Glu-	— " —
7	— " —	— " —	-Asp-**His**(111)-Glu-	— " —
8	— " —	— " —	-Asp-**Ala**(111)-Glu-	— " —
9	— " —	— " —	-Asp-**Leu**(111)-Glu-	— " —
10	-Pro-**Glu**(112)-Asn-	— " —	-Pro-**Lys**(112)-Asn-	— " —
11	— " —	— " —	-Pro-**Gln**(112)-Asn-	— " —
12	— " —	— " —	-Pro-**Asp**(112)-Asn-	— " —
13	-Leu-**Gln**(109)-**Asp**(110)-**Pro**(111)-Glu-	deletion	-Leu(108)-Glu(112)-	no

mutant proteins with the same electrophoretic mobility as the *Eco*RII endonuclease from *E. coli* JM109 carrying the pR224H plasmid. Tests on the ability to specifically cleave a standard DNA revealed *Eco*RII endonuclease activity in all cell extracts of *E. coli* JM109 strains carrying all substitution variants for all amino acid residue codons (table). In contrast, there was no *Eco*RII endonuclease activity in the cell extract from the *E. coli* JM109 strain carrying a plasmid with the deletion in the *ecoRII* gene.

Thus, 12 mutants resulting from site-directed mutagenesis have been characterized that have codon substitutions in a distinct site of the *ecoRII* gene. The expression of the mutant genes in *E. coli* cells resulted in mutant *Eco*RII proteins with point amino acid substitutions: Asp(110) → Lys, Asn, Thr, Val, or Ile; Pro(111) → Arg, His, Ala, or Leu; Glu(112) → Lys, Gln, or Asp. All these proteins demonstrated *Eco*RII endonuclease activity. We can exclude the accidental preservation of catalytic activity because the amino acid residues in each position were replaced by different ones possessing different and in some cases opposite properties. Thus, it is reasonably safe to conclude that the Asp110–Glu112 region of the *Eco*RII enzyme does not belong to the catalytic site. Moreover, none of the residues Asp110, Pro111, and Glu112 is directly involved in formation of this site. However, the experiment on the deletion of the codons for amino acid residues 109-111, resulting in complete loss of *Eco*RII endonuclease activity, suggests that this region plays an important role in formation and/or maintenance of the tertiary structure of the *Eco*RII restriction endonuclease.

Thus, the question of which amino acid residues are involved in formation of the *Eco*RII restriction endonuclease active site remains unsolved. However, it was recently reported that mutant E96A of *Eco*RII endonuclease (mutation in the region of the P76 D77 X_{18} E96 K97 motif) lacks the ability to bind and cleave DNA [12]. This report and ours narrow the boundaries of where the active site may be found and allow us to plan further studies on the mutagenesis of *Eco*RII. These studies are currently in progress in our laboratory.

We are grateful to professor A. Bhagwat (Wayne State University, Detroit, MI, USA) for providing us with the pR224 plasmid. This study was supported by the Howard Hughes Medical Institute (grant HHMI 75195-545501), the Russian State Scientific Program "Newest Methods in Bioengineering", direction "Protein Engineering" (project No. 3-07), and the Russian Foundation for Basic Research (grant No. 98-04-49108).

REFERENCES

1. Roberts, R. J., and Macelis, D. (1998) *Nucleic Acids Res.*, **26** , 338-350.
2. Aggarwal, A. K. (1995) *Curr. Opin. Struct. Biol.*, **5**, 11-19.
3. Pingoud, A., and Jeltsch, A. (1997) *Eur. J. Biochem.*, **246**, 1-22.
4. Anderson, J. E. (1993) *Curr. Opin. Struct. Biol.*, **3**, 24-30.

5. Wah, D. A., Hirsch, J. A., Dorner, L. F., Schildkraut, I., and Aggarwal, A. K. (1997) *Nature*, **388**, 97-100.
6. Wah, D. A., Bitinaite, J., Schildkraut, I., and Aggarwal, A. K. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 10564-10569.
7. Kovall, R. A., and Matthews, B. W. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 7893-7897.
8. Skirgaila, R., Grazulis, S., Bozic, D., Huber, R., and Siksnys, V. (1998) *J. Mol. Biol.*, **279**, 473-481.
9. Krueger, D. H., Kupper, D., Meisel, A., Reuter, M., and Schroeder, C. (1995) *FEMS Microbiol. Rev.*, **17**, 177-184.
10. Petrauskene, O. V., Karpova, E. A., Gromova, E. S., and Guschlbauer, W. (1994) *Biochem. Biophys. Res. Commun.*, **198**, 885-890.
11. Topal, M. D., and Conrad, M. (1993) *Nucleic Acids Res.*, **21**, 2599-2603.
12. Reuter, M., Schneider-Mergener, J., Kupper, D., Meisel, A., Mackeldanz, P., Krueger, D. H., and Schroeder, C. (1999) *J. Biol. Chem.*, **274**, 5213-5221.
13. Drutsa, V. L., Krivonos, A. V., Koroleva, O. N., and Shabarova, Z. A. (1990) *Bioorg. Khim.*, **16**, 1052-1059.
14. Bhagwat, A. S., Johnson, B., Weule, K., and Roberts, R. J. (1990) *J. Biol. Chem.*, **265**, 767-773.
15. Kossykh, V., Repyk, A., Kaliman, A., and Buryanov, Y. (1989) *Biochim. Biophys. Acta*, **1009**, 290-292.
16. Drutsa, V. L., and Kaberdin, V. R. (1992) *Nucleic Acids Res.*, **20**, 922.
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1981) *Molecular Cloning*, Cold Spring Harbor, N. Y.
18. Selent, U., Rueter, Th., Koehler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschlaeger, Th., Wolfes, H., Peters, F., and Pingoud, A. (1992) *Biochemistry*, **31**, 4808-4815.