Summary of Ph.D. thesis

Analysis of certain aspects of restriction endonuclease function: DNA recognition and *in vivo* effects

Tetiana Ivanenko

Supervised by Antal Kiss

Institute of Biochemistry
Biological Research Center
Hungarian Academy of Sciences

Szeged
2002

Introduction

The PhD thesis comprises three related projects. The first study is a mutational analysis of DNA substrate recognition by the *Eco*RI endonuclease. The second deals with an *in vivo* effect of restriction enzymes. The third, as yet unfinished, project explores the possibility of constructing chimeric restriction endonucleases and analyzes the phenotypes of one of the constructs. The common theme in all three projects is sequence-specificity of type II restriction endonucleases.

I. DNA substrate recognition by the *Eco*RI endonuclease

*Eco*RI endonuclease is one of the best characterized restriction enzymes and a widely used biochemical tool. It recognizes the sequence GAATTC in double stranded DNA and cleaves both strand between the G and A. The enzyme requires Mg^{2+} as cofactor for cleavage, but can specifically bind to its cognate sequence in the absence of Mg^{2+}. Its counterpart, the *Eco*RI methylase recognizes the same sequence and transfers one methyl group to the internal adenines in both strands of the target sequence. The methylated recognition sequence is resistant to *Eco*RI endonuclease.

*Eco*RI endonuclease is a homodimer composed of two 277-residue polypeptides of MW = 31.063. *Eco*RI endonuclease was the first type II restriction enzyme for which a crystal structure of the complex between the protein and the cognate oligonucleotide became available. The co-crystal structure of the *Eco*RI-DNA complex identified several amino acids that appeared to be in sequence-specific contact with the bases of the recognition sequence. A thorough understanding of the molecular mechanism underlying sequence specificity requires the use of complementary approaches to test the predictions of the crystal structure. A number of these contacts, mainly those to purines of the recognition sequence, were previously tested by mutagenesis and biochemical studies. Less attention has been given to interactions with pyrimidines. It has been proposed, that the cytosine of the target sequence interacts with three residues: the amino group

donates a hydrogen bond to the main chain carbonyl of A138 and H5 and H6 make van der Waals contacts with M137 and I197. The role of A138 in substrate recognition was supported by genetic studies.

II. DNA single-strand break repair in *E.coli*

E.coli has a sophisticated machinery to repair many types of DNA damage. One type of DNA damage is the single-strand break (nick) that should be relatively benign because the intact DNA strand holds together the ends of the severed strand.

Our collaborator, J. Heitman isolated the *Eco*RI endonuclease mutants E144C and R200K. The mutants had reduced endonuclease activity and retained their specificity for the wild-type *Eco*RI recognition site. When tested *in vitro*, both mutants were found to predominantly nick only one strand of the DNA substrate. The mutant enzymes displayed temperature-sensitive activity. Expression of these *Eco*RI endonuclease mutants in the absence of the *Eco*RI methyltransferase was found to induce the SOS DNA repair response and greatly reduce viability of *E. coli* hosts deficient in RecA and RecB functions. This was a surprising observation and suggested that DNA ligase by itself might not always be sufficient to repair single-strand breaks. It looked important to test how general this phenomenon was. To complement Heitman's studies, we chose another experimental system to generate nicks and test their effects in an *E. coli* host. The approach we used was based on the observations by Halford and coworkers. They found that the *Eco*RV endonuclease, when overproduced in an m+ host, makes nicks at noncognate sites.

III. *Eco*RI shares structural and functional similarities with its isoschizomer *Rsr*I

*Rsr*I endonuclease is an isoschizomer of *Eco*RI, i.e. it recognizes the sequence GAATTC and cleaves it between the G and A residues. Methylation of the substrate sequence by M·*Eco*RI protects the site from cleavage by either enzyme. Like the *Eco*RI endonuclease, the *Rsr*I endonuclease exists as a dimer in

solution and requires Mg^{2+} cofactor for catalysis. There is a more than 50% amino acid sequence identity between *Eco*RI and *Rsr*I. More importantly, all amino acids that are known to mediate sequence-specific recognition and catalysis in *Eco*RI are conserved in *Rsr*I, suggesting that *Rsr*I uses the same mechanism as *Eco*RI to interact with its DNA substrate.

In substrate-enzyme complexes, both *Eco*RI and *Rsr*I were found to footprint 12 base pairs, bend DNA by 50°, and unwind the DNA helix by 25°. Based on these observations it was concluded that the two enzymes interact with their recognition sequences in similar way. Other experiments identified differences in the DNA recognition mechanisms of *Eco*RI and *Rsr*I. *Rsr*I endonuclease appeared to be more sensitive to alterations of the functional groups within its recognition sequence than *Eco*RI. The sequence homology between *Eco*RI and *Rsr*I endonucleases suggested to us that it could be possible to construct active hybrids between them. Making the hybrids between these two endonucleases we wished to test the structural and functional relatedness of *Eco*RI and *Rsr*I.

Specific Aims

- To test the role of two amino acids (I197 and M137) which were predicted on basis of the X-ray crystal structure to mediate DNA recognition by *Eco*RI endonuclease.
- To explore the repair pathway of DNA single strand breaks inflicted by restriction endonucleases.
- To construct *Eco*RI/*Rsr*I recombinant endonucleases.

Results and Conclusions

I. DNA substrate recognition by the *Eco*RI endonuclease

- *Site-directed mutagenesis*

To facilitate genetic manipulation of the *Eco*RI endonuclease, we used a two-plasmid system in which the *Eco*RI endonuclease and methyltransferase genes were cloned on compatible plasmids: pJH15b^{TS6} and pJC11. Substitutions at M137 and I197 were introduced by oligonucleotide-mediated *in vitro* mutagenesis. Temperature sensitivity of the *Eco*RI variant encoded by pJH15b allowed us to test the effects of the mutations in the absence of the *Eco*RI methyltransferase. *Eco*RI activity was assessed by measuring phage restriction, testing cell viability and SOS induction.

- *Substitutions at M137*

A total of eight substitutions at M137 were identified: G, A, V, W, R, P, T, K.

a) None of the mutants exhibited restriction;
b) None of the crude extracts prepared from the mutants showed detectable *Eco*RI activity under the conditions used;
c) M137G, A, V and T were inviable in the absence of *Eco*RI methyltransferase, indicating that active *Eco*RI endonuclease was produced and damaged host cell DNA. M137R grew very slowly and developed dark blue color on X-gal indicator medium as a sign of SOS induction. M137W, P and K were inactive;

- *Substitutions at I197*

Ten different substitutions at I197 were isolated: V, L, M, G, Q, A, W, R, D, P.

a) Replacements L, M had no effect on the level of the phage restriction compared to WT, whereas others (G, A, Q, R, W) led to a 10- to 100-fold reduction. I197D, P completely abolished restriction. I197V substitution caused a 10-fold increase in restriction.
b) The *Eco*RI activity detected *in vitro* in crude extracts largely paralleled data from phage restriction assay.

c) All I197 mutants were inviable in the absence of *Eco*RI methylase.

Conclusions

1) None of the replacements at M137 or I197 resulted in altered specificity;
2) The observation that substitutions at M137 lead to a severe loss of activity is consistent with the role predicted for M137;
3) I197 is unlikely to be directly involved in substrate recognition by the *Eco*RI endonuclease;

II. Repair of *Eco*RV endonuclease-inflicted DNA single-strand breaks is RecA- and RecB dependent

An *Eco*RV overexpression system was used to generate nicks in *E.coli* DNA *in vivo.* The system consisted of two plasmids, pMetB and pBSKSRVD, which endode the *Eco*RV methyltransferase and endonuclease, respectively. Expression of the *Eco*RV endonuclease in this system was controlled by the temperature-sensitive (*cI857*) λ repressor. It was known that the *Eco*RV endonuclease, when present at high concetrations in the cells, makes single-strand cuts at sites which differ from the canonical site by one nucleotide and are not protected by the cognate methylation.

To test the involvement of different mechanisms in the repair of *Eco*RV-inflicted nicks, the plasmids pMetB and pBSKSRVD were introduced into an isogenic set of repair-defective strains. The *in vivo* effect of *Eco*RV endonuclease was assayed by determining the number of viable cells at 30°C and 42°C.

a) The WT and *recN262* mutant strains showed similar colony-forming efficiency at both temperatures, indicating that they were not sensitive to increased *Eco*RV endonuclease activity. In contrast, the viability of the *recA56* and *recB21* strains was at least five orders of magnitude lower at 42°C, than at 30°C. Bacteria that carried pMetB alone, or pMetB plus the control plasmid expressing an inactive *Eco*RV endonuclease, showed the same colony-forming efficiency and morphology at both the repressing (30°C) and inducing (42°C) temperatures.

b) The *lexA3* mutant strain exhibited slower growth and altered morphology (unequal size, irregular edges and shape) at the inducing temperature (42°C), indicating that *Eco*RV endonuclease overexpression was deleterious to *lexA3* mutant cells.

To test whether the damage made by *Eco*RV endonuclease can be prevented by increasing DNA ligase activity in cell, viability assays were performed with cells that contained plasmid pMetBLG1, which is pMetB carrying the *E.coli* ligase gene.

a) It was shown that *Eco*RV overexpression was not lethal to *recA56* and *recB21* cells when ligase level was elevated.
b) The beneficial effect of the extra copies of the *lig* gene was also evident from the phenotype of the *lexA3* host, in which the colonies exhibited normal growth rate and colony morphology.

Conclusions

1) Repair of *Eco*RV-inflicted nicks required RecA and RecB functions and was at least partially dependent on the SOS response.
2) The balance between nicks and ligase capacity is critical in determining the mechanism by which single-strand DNA breaks are repaired.
3) The toxicity of *Eco*RV inflicted nicks can be explained in a frame of Kuzminov model.

III. Construction of *Eco*RI-*Rsr*I recombinant endonucleases

Five *Eco*RI-*Rsr*I hybrid proteins were constructed by replacing segments of the *ecoRIR* gene with the corresponding, PCR-amplified segments of the *rsrRIR* gene. The segments were determined by three unique restriction sites (*Hin*dIII, *Bgl*II, *Pst*I) in the *ecoRIR* gene. The chimeric proteins were named according to their structure with regard to the origin of the fused parts: EREE, EERE, ERRE, REEE, EEER (E, *Eco*RI-derived segment, R, *Rsr*I-derived segment).

- *In vivo characterization of the EcoRI-RsrI recombinant proteins*

a) Recombinant endonucleases ERRE, REEE, EEER and EREE proved inactive at all temperatures tested, by the criteria that *E.coli* JH140 cells, which contained the plasmid with the hybrid endonuclease gene, but did not produce *Eco*RI methylase, were viable.

b) Cells with the EERE construct were viable only when *Eco*RI methylase was present, indicating that the EERE recombinant endonuclease was active.

- *In vivo characterization of the EERE hybrid protein*

a) EERE exhibited temperature-sensitive phenotype which facilitated study of its properties *in vivo*.

b) Expression of EERE induced the SOS response.

c) To see whether the observed *in vivo* effect was dependent on a particular genetic background, we tested other host strains. Expression of EERE in RR1 (K12/B hybrid strain) was lethal at permissive temperature (30°C) whereas expression of the parental *Eco*RI^{TS6} just slowed growth without significant drop in viability. HB101 (*recA* derivative of RR1) was more sensitive to EERE than RR1, which suggested that DNA damage caused by EERE requires DNA recombination for repair.

d) *recA56*, *recB21* and *lexA3* strains were more sensitive to EERE than the WT parental strain.

e) EERE did not restrict unmodified λ_{vir} phage. No restriction could be achieved even if EERE was overproduced in the cell.

f) We compared the level of genomic DNA degradation in cells expressing EERE or *Eco*RI^{TS6} and monitored cell viability. The viable cell number dropped sharply in both cultures, i.e. cultures expressing either *Eco*RI^{TS6} endonuclease or the EERE hybrid. However, total DNA purified from *Eco*RI^{TS6}-producing cells showed extensive degradation, whereas DNA extracted form EERE-producing cells was much less degraded.

- *In vitro characterization of EERE*

Crude extracts prepared from JH140 (EERE) and JH140 (*Eco*RI^{TS6}) grown at 30°C were assayed for restriction enzyme activity using *Eco*RI and *Rsr*I reaction buffers.

a) No specific endonuclease activity was detected in the JH140 (EERE) extracts, whereas a 10-fold diluted extract prepared from JH140 (*Eco*RI^{TS6}) yielded almost complete digestion of λ DNA.

b) A plasmid overexpressing EERE was constructed by cloning the EERE gene in the plasmid pER23S(-ATG). In this plasmid the EERE gene is transcribed from the *rrnB* P_2 promoter. EERE was partially purified. When, during purification, fractions were assayed for endonuclease activity, we observed electrophoretical retardation of plasmid DNA pUC18 (has one *Eco*RI site). Western blot analysis revealed the presence of EERE in those fractions that caused slower migration of pUC18 during electrophoresis. It was also shown that the observed electrophoretical retardation was dependent on the presence of *Eco*RI site in the plasmid. Preliminary data indicate that EERE can cleave supercoiled pUC18 and pUC19 at a very low rate.

With the aim to purify EERE by affinity chromatography, we have constructed two plasmids (pET3-His-EERE and pFLAG-MAC-EERE), which encode EERE variants with an N-terminal affinity tag.

Conclusions

1) Repair of the DNA damage caused by the *Eco*RI-*Rsr*I hybrid protein EERE requires homologous DNA recombination.
2) Greater toxicity of the EERE protein compared to that of *Eco*RI^{TS6} suggest that the DNA lesions caused by EERE might be different from *Eco*RI double-strand cuts.
3) We suppose that the toxic phenotype of EERE results from its tight binding to *Eco*RI sites and that EERE kills the cell by interfering with transcription and/or replication.

List of publications

1. Ivanenko, T., Heitman, J. and Kiss, A. (1998). Mutational analysis of the function of Met137 and Ile197, two amino acids implicated in eequence–specific DNA recognition by the *Eco*RI endonuclease. Biological Chemistry, *379*: 459 – 465.
2. Heitman, J., Ivanenko, T. and Kiss, A. (1999). DNA nicks inflicted by restriction endonucleases are repaired by a RecA– and RecB–dependent pathway in *Escherichia coli*. Mol. Microbiol. *33*, 1141 – 1151.

Presentations at scientific meetings

1. Ivanenko, T., Heitman, J. and Kiss, A.: "Role of Met137 and Ile197 in sequence specific recognition by the EcoRI endonuclease" Second Molecular Biology Workshop of the Hungarian Biochemical Society, May, 1997, Lillafured, Hungary (talk).
2. Ivanenko, T., Heitman, J. and Kiss, A.: "Role of Met137 and Ile197 in sequence specific recognition by the EcoRI endonuclease" 4th New England Biolabs Workshop on Biological DNA Modification, September 2 – 7, Igls, Austria. (poster).
3. Ivanenko, T., Heitman, J., Kiss,A.:"Repair of ssDNA breaks inflicted by restriction endonucleases in *E.coli*", Straub Days, January 20-22, 1999, BRC, Szeged, Hungary (talk).
4. Heitman, J., Ivanenko, T. and Kiss, A.:" DNA nicks inflicted by restriction endonucleases are repaired by a RecA – and RecB – dependent pathway in *Escherichia coli*" Howard Hunges Medical Institute Meeting of International Research Scholars, June 22 – 25, 1999, Moscow, Russia, (talk).
5. Ivanenko, T., Heitman, J. and Kiss, A.: "Construction of hybrids between the EcoRI and RsrI restriction endonucleases" Howard Hunges Medical Institute Meeting of International Research Scholars, June 20 – 23, 2000, Chevy Chase, Maryland, USA (talk).