Mutational Analysis of Two Putative Catalytic Motifs of the Type IV Restriction Endonuclease Eco57I

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The role of two sequence motifs (SM) as putative cleavage catalytic centers 77PDX13EAK (SM I) and 811PDX29DKQ (SM II) of type IV restriction endonuclease Eco57I was studied by site-directed mutational analysis. Substitutions within SM I; D78N, D78A, D78K, and E92Q reduced cleavage activity of Eco57I to a level undetectable both in vivo and in vitro. Residual endonuclease activity of the E92Q mutant was detected only when the Mg2+ in the standard reaction mixture was replaced with Mn2+. The mutants D78N and E92Q retained the ability to interact with DNA specifically. The mutants also retained DNA methylation activity of Eco57I. The properties of the SM I mutants indicate that Asp78 and Glu92 residues are essential for cleavage activity of the Eco57I, suggesting that the sequence motif 77PDX13EAK represents the cleavage active site of this endonuclease. Eco57I mutants containing single amino acid substitutions within SM II (D812A, D833N, D833A) revealed only a small or moderate decrease of cleavage activity as compared with wild-type Eco57I, indicating that the SM II motif does not represent the catalytic center of Eco57I. The results, taken together, allow us to conclude that the Eco57I restriction endonuclease has one catalytic center for cleavage of DNA.

Nearly 3000 restriction endonucleases with over 200 different specificities, which together with cognate DNA methyltransferases constitute restriction-modification (R-M) systems, have been identified in bacteria (1). Restriction-modification enzymes are traditionally divided into three classes designated type I, II, and III on the basis of enzyme subunit composition, cofactor requirements, substrate specificity characteristics, and reaction products (2). An increasing number of restriction endonucleases that do not fit into the conventional classification however have been reported (3–7). Their differences from the type I and type III enzymes are so substantial that a classification as new kinds of restriction endonucleases; type IIS, type IIT, type IV, and Bcg-like has been suggested (3–8).

The type IV restriction endonuclease Eco57I has been studied in detail (4). Similar to type IIS endonucleases, it recognizes an asymmetric nucleotide sequence, cleaves both DNA strands outside the target site 5'-CTGAAG(N)10-3', and exists in solution as a monomer. Other features of Eco57I, however, such as stimulation of endonucleolytic reaction with the DNA methyltransferase cofactor S-adenosyl-l-methionine (AdoMet) and methylation of one strand of the recognition duplex, makes it similar to type III enzymes. Both endonucleolytic and methylation activities reside within a single large polypeptide of the enzyme. In addition to the bifunctional restriction endonuclease, the Eco57I R-M system also includes a separate Eco57I methyltransferase, which modifies both DNA strands of the target duplex. The methylation domain has been previously assigned to the carboxyl-half of the Eco57I restriction endonuclease, where conserved amino acid sequence motifs typical for m6A DNA methyltransferases involved in AdoMet binding and catalysis of methyl group transfer are located (9). The location and identity of the endonuclease active center though remains to be determined and is addressed here.

In contrast to DNA methyltransferases, the amino acid sequences of restriction endonucleases share little similarity. This observation therefore reduces the possibility of identifying catalytic sites of restriction enzymes on the basis of sequence alignment. Structural and mutational analysis of type II restriction endonucleases revealed however the PDX13/D/E/XK motif as a catalytic/Mg2+ binding signature motif (8, 10, 11). Two putative catalytic/Mg2+ binding motifs (i.e. 77PDX13EAK and 811PDX29DKQ, located in the N-terminal and C-terminal parts of Eco57I, respectively) have been described in the amino acid sequence of the enzyme (10). The statistical significance of these motifs however is low, and their presence does not allow unambiguous prediction of the active site, as is evidenced by the following observations. (i) Cfr10I contains the PDX13/D/E/XK motif, but it is not part of its catalytic center (12) and (ii) EcoRI contains two such motifs, one of which is not involved in catalysis (10).

On the other hand it cannot be excluded that two active centers are necessary for monomeric Eco57I to cleave both DNA strands. The asymmetric nature of the Eco57I target sequence is inconsistent with the use of a symmetric dimer for recognition and DNA cleavage, as in the type II restriction endonucleases. A single molecule containing two endonucleolytic centers could cleave both DNA strands. It has been suggested that a molecule recognizing an asymmetric nucleotide sequence with a single catalytic center must rearrange the catalytic center for sequential cleavage of each DNA strand, or it must form a higher order complex to cleave both strands of DNA (13). This second mechanism is utilized by the restriction endonuclease FokI, the only type IIS enzyme characterized in this respect so far (14). The identification of the Eco57I catalytic center(s) would increase our understanding of the functional organization of a unique enzyme, which shares proper-
ties with type IIS and type III enzymes. We therefore constructed single amino acid substitutions in the putative catalytic motifs of Eco57I to determine the role of the two putative active centers, if any, in DNA cleavage. The properties of the mutants suggest that of the two putative catalytic motifs, only the motif $^{33}$PDX$_3$EAK is involved in DNA cleavage catalysis.

### Experimental Procedures

#### Bacterial Strains, Plasmids, Phage, and Media—The Escherichia coli strain ER2267 was used as host for cloning procedures and was used to express the endonuclease activity of the wt Eco57I and mutants in vivo. It was kindly provided by E. Raleigh. The E. coli CJ236 strain of genotype $^{F \text{-}}$ cat ($= pCj105; M_{33}^{\text{Cm}}; $) dcat ung1 thi-1 relA1 spoT1 mcra was used to prepare single-stranded DNA for site-directed mutagenesis. The E. coli BL21 strain (Novagen) was used for the expression of the wild-type eco57IR and mutant genes. The pUC19-based phagemid pTZ19IR (15) was used as a vector in site-directed mutagenesis experiments and DNA sequencing. The plasmid pEco57IR3.6 (Ap$^+$) was constructed by subcloning eco57IR from pEco57IRM6.3 (9) into the pET-21b expression vector (Novagen) in the orientation coinciding with that of the T7 promoter. It was used for the construction of mutant eco57IR genes and for expression of purpose for protein purification.

Plasmid pEco57IR3.6 (Ap$^+$) carries the om57I methyltransferase gene cloned in the vector pACYC184 (9). Transformation of E. coli was carried out by the CaCl$_2$ heat shock method (16). All strains were grown in Luria-Bertani medium at 37 °C. The following concentrations of antibiotics were used when necessary: ampicillin (Ap), 60 μg/ml; kanamycin (Km), 50 μg/ml; chloramphenicol (Cm), 30 μg/ml; $\lambda_{\text{nu}}$, was used to test the in vivo function of the wild-type Eco57I and mutants. Stocks of this phage were prepared according to Sambrook et al. (16).

#### Enzymes, Chemicals, and Oligonucleotides—All enzymes, including a homogenous preparation of wt Eco57I, kits, and λ DNA were provided by MBI Fermentas and used according to the manufacturer's recommendations. [α-$^{32}$P]dATP and [γ-$^{32}$P]dATP were purchased from Amersham Pharmacia Biotech. Synthetic oligonucleotides were synthesized at the facilities of MBI Fermentas. All other chemicals were reagent grade commercial products.

#### Analysis of Viability of Strains Containing Wt and Mutant Eco57I—The ability of strains harboring wt or mutant Eco57I to survive in the presence or absence of Eco57I methylase was tested by transforming E. coli strain ER2267 containing or lacking pEco57IRM3.3 with plasmids carrying either wt or mutant eco57IR genes. An aliquot of 0.5 μg of each plasmid DNA in a total volume of 1 ml was transformed to a 100-μl aliquot of competent cells. Ap$^+$ or Ap$^+$Cm$^+$ transformers were selected. The transformation efficiency of competent cells was tested by transforming them with the control plasmid pBR322. Two independent transformation experiments were carried out.

#### DNA Preparation and Manipulation—Plasmids were prepared by the alkaline lysis procedure (17) and purified additionally as described by Maniatis et al. (20). Bacterial DNA was isolated by the alkaline lysis method (16). Restriction plasmid construction and isolation of DNA fragments from agarose gels were performed according to standard techniques (16). DNA sequencing was carried out by the chain termination method (19).

#### Site-directed Mutagenesis—To generate the single-stranded DNA needed for mutagenesis, a 180-bp Eco88I-Ber1107I and 680-bp PstI-Eco105I DNA fragments of the eco57IR gene (GenBank/EMBL accession no. X61122) containing N-terminal (SM I) and C-terminal (SM II) putative catalytic motifs, respectively were subcloned into the pTZ19IR phagemid, which was then multiplied in the CJ236 strain. Site-directed mutants were obtained by oligonucleotide mutagenesis using the method of Kunkel et al. (20). Eco57I mutant proteins that differ from the wild type by only a single amino acid were made using the corresponding oligonucleotides: D78N, T78AAGGCCCAACACTACGGC, D78A, TAAAAGCCACGCTTACGCT, D78K, TAAAAAGCCAAGATCACTGGT, E92Q, TTTTCTTCTACGGCCAA, D812A, TATTAGCGGCC-CCACTGGC, D833N, CTGGATGTTAGCCAGAGCT, D833A, CTG-CAATCCGCGCAAGCTT. After introducing changes verified by DNA sequencing, the fragments of the eco57IR containing desired point mutations were exchanged with the corresponding fragments of the wild-type Eco57I in vivo. The ability of subcloning sites was checked by restriction analysis.

#### Gel Electrophoresis of Proteins—One-dimensional gel electrophoresis of proteins under denaturing conditions was performed as previously described (21). SDS-PAGE was carried out on a 7.5% separating gel. Protein bands were visualized after Coomassie Blue R250 staining.

#### Purification of Mutant and Wild-type Eco57I Endonucleases—E. coli BL21, freshly transformed by the plasmid containing one of the mutant eco57IR genes, was used as the source of the mutant enzyme. Expression was induced by adjusting the culture to 1 mM isopropyl-1-thio-β-D-galactopyranoside at an $A_{500}$ of about 0.6. After 3 h, the cells were chilled on ice, harvested by centrifugation and stored at −80 °C. All enzymes were stored at 10 mM MgCl$_2$, 0.1 mM DTT, 7 mM β-mercaptoethanol containing 0.1 mM NaCl. Cells were disrupted by sonication and cell debris was removed by centrifugation. The supernatant was applied to a Heparin-Sepharose column (1.5 × 30 cm) equilibrated with buffer A containing 0.1 mM NaCl. The column was washed with the same buffer and eluted with a 400-ml linear gradient of 0.1–1.0 mM NaCl in buffer A. Fractions containing the mutant Eco57I protein, as determined by SDSPAGE gel electrophoresis, eluted at −0.28–0.44 mM NaCl. They were pooled and dialyzed against buffer A containing 0.05 mM NaCl and applied to a Sepharose Q column (1.5 × 20 cm). The column was washed with the same buffer and eluted with a 340-ml linear gradient of 0.05–0.3 mM NaCl in buffer A. The peak fractions, which eluted at −0.12–0.17 mM NaCl were pooled and dialyzed against 0.1 mM NaCl and applied to an AH-Sepharose column (1.5 × 9 cm). After the column was washed with buffer A, sample was eluted with a 200-ml linear gradient of 0.1–1.0 mM NaCl in buffer A. The peak fractions (eluted at 0.31–0.38 mM NaCl) were pooled, dialyzed against the storage buffer (10 mM potassium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA, 7 mM β-mercaptoethanol, and 50% glycerol) and stored at −20 °C. Essentially the same procedure was used for purification of wt Eco57I, which was kindly provided by MBI Fermentas. The proteins were homogeneous as judged by polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 120,390 M$^{-1}$ cm$^{-1}$ for a monomer calculated from the amino acid composition (22). The concentrations of Eco57I are given in terms of the monomeric protein.

#### DNA Cleavage Assay—The endonuclease activity in vitro was tested by incubation of serial dilutions of purified proteins or cell-free extracts prepared as previously described (23) with 1 μg of λ DNA at 37 °C for 1 h in a 50-μl reaction volume containing 10 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 0.01 mM AdoMet, 0.1 mg/ml bovine serum albumin (standard reaction mixture), followed by electrophoresis on 0.8% agarose gels. The same reaction buffer was used for determination of specific activity of purified wt or mutant Eco57I. The amount required to hydrolyze 1 μg of λ DNA in 1 h at 37 °C until no change in the cleavage pattern was observed. In some experiments the standard reaction mixture was modified to include MnCl$_2$ instead of MgCl$_2$ and Sinefulgin instead of AdoMet.

#### In vivo activity of wt and mutant restriction endonucleases was tested by comparison of plating efficiency of the λnu bacteriophage on ER2267 carrying wt Eco57I or mutants of Eco57I methyltransferase on the compatible plasmid pEco57IRM3.3, to that on ER2267 expressing only Eco57I methylase (nonrestricting host). Portions of serially diluted phage stock were spotted on a lawn of bacteria, and the plates were incubated at 37 °C. The phage titer was determined (16). The efficiency of plating (e.o.p.) was defined as the phage titer on the host under investigation divided by the phage titer on a nonrestricting host.

#### DNA Methylation Assay—The modification activity in vitro was tested by the DNA protection assay where 1 μg of λ DNA served as substrate in 50 μl of reaction mixture (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mM AdoMet). The reaction was initiated by the addition of varying amounts of sample solution and incubated for 1 h at 37 °C. The reaction was terminated by heating at 65 °C for 20 min. MgCl$_2$ solution (final concentration of 10 mM), and an excess of Eco57I was then added to the reaction mixture. The incubation was continued for 1 h at 37 °C. The reaction products were resolved by agarose gel electrophoresis. 1 unit of the modification activity was defined as the amount of enzyme that in 1 h at 37 °C could methylate 1 μg of DNA resistant to the enzyme by Eco57I.

#### Gel Electrophoresis of DNA—Electrophoresis of DNA was performed on 0.7% agarose gel. DNA was prepared from 210-bp EcoRI-HindIII DNA fragment excised from pEco57IRM6.3 containing a single Eco57I site in the middle of the sequence was used as the specific DNA fragment. It was cloned into the phagemid pTZ19IR, and a single-nucleotide substitution was introduced using a mispaired oligonucleotide by the method of Kunkel et al. (20), generating a PstI site instead of the Eco57I site. The resultant nonspecific 210-bp DNA...
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### RESULTS

#### Analysis of the Eco57I amino acid sequence revealed two putative catalytic/Mg$^{2+}$ binding sites: sequence motifs $^{7}$PD$^{13}$_EAK (SM I) and $^{11}$PD$^{29}$DQK (SM II) (10). To assess their relevance, if any, to cleavage activity of the enzyme, we constructed a range of single amino acid substitution mutants of the acidic residues of the motifs; the residues most conserved in catalytic/Mg$^{2+}$ binding centers of restriction endonucleases (8, 11). The following mutants were constructed by site-directed mutagenesis: D78N, D78A, D78K, E92Q for SM I and D812A, D833A, D833N for SM II. The mutants were analyzed for their cleavage, methylation, and DNA binding activities.

### Enzymatic activities of wild-type Eco57I and mutants in vivo and in vitro

| Enzyme | Survival in Eco57I methylase-deficient cells $^a$ | Efficiency of plating of λvir $^b$ | Cleavage activity in crude cell lysates $^c$ | Specific cleavage activity $^d$ | Specific methylation activity $^e$ |
|--------|--------------------------------------------|-----------------|----------------------------------------|-------------------|---------------------|
| wt Eco57I | − | 2.6 x 10$^{-5}$ | + | 2800 | unit/mg protein $^a$ |
| D78N | + | 1 | + | 2500 | |
| D78A | + | 0.9 | − | NA $^c$ | |
| D78K | + | 1 | − | NA | |
| E92Q | + | 0.8 | − | NA | |
| D812A | − | 2.8 x 10$^{-5}$ | + | 2400 | |
| D833A | − | 3.7 x 10$^{-5}$ | + | 1800 | |
| D833N | − | 3.2 x 10$^{-5}$ | + | 1100 | |

$^a$ Two independent transformation experiments were carried out. −, no transformants; +, effective transformation.

$^b$ All determinations were carried out at least in triplicate. The efficiency of plating was defined as the phage titer on the host under investigation divided by the phage titer on the non-restricting host (carrying only Eco57I methylase).

$^c$ −, no activity; +, endonucleolytic activity was detectable.

$^d$ Specific activities were determined as the average of three independent measurements. −, no activity.

$^e$ NA, not assayed.

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The fragment was excised from the phagemid with EcoRI and HindIII restriction endonucleases. Both specific and nonspecific fragments were gel purified and radiolabeled using Klenow polymerase to fill in their 5’ extensions with [α-32P]dATP and the other three dNTPs (16). DNA fragment was dissolved spectrophotometrically.

**Gel Mobility Shift Assay**—Binding reactions (20 μl) contained the 32P-end labeled- specific or -nonspecific DNA fragment (final concentration 10 pm) and the wt Eco57I or mutant protein (final concentration in the range of 0–10 nM). The incubation was performed in binding buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM CaCl2, 0.1 mg/ml bovine serum albumin, 10% glycerol) for 20 min at room temperature, and then the samples were applied to a 6% polyacrylamide gel (29:1 acrylamide/bis). Electrophoresis was carried out at 11 V/cm at room temperature in the gels were dried, and radioactive bands were visualized using the OptiQuant™ Image Analysis Software (Pacard).

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In vitro cleavage activity correlated well enough with the restriction activities observed in vivo to suggest that amino acids Asp-78 and Glu-92 are essential for the endonucleolytic activity of the Eco57I restriction endonuclease, whereas Asp-812 and Asp-833 of SM II are not. To test whether this effect was caused by the loss of ability to cleave DNA by the D78N and E92Q mutants or a deficiency in specific DNA binding, an electrophoretic mobility shift assay was used for characterization of the mutants as compared with wild-type Eco57I.
**Binding of Wt and D78N, E92Q Mutant Proteins to the Eco57I Target Site**—The effects of mutations on binding of the mutant Eco57I proteins to DNA were examined using the gel mobility shift assay. Two DNA fragments were used: a 210-bp DNA fragment containing one Eco57I site in the middle of the sequence (specific DNA) and a nonspecific DNA fragment, which had the same sequence, except it lacked the Eco57I site as a result of a 1-bp substitution. The experiments were performed with purified wild-type Eco57I, D78N, and E92Q mutant proteins. In initial experiments, the DNA binding for the wild-type Eco57I restriction endonuclease was characterized. Increasing amounts of the Eco57I endonuclease were added to a fixed amount of $^{32}$P-end labeled specific or -nonspecific DNA fragment, and the free DNA was separated by electrophoresis from the DNA complexed with the protein. Several attempts to visualize specific Eco57I-DNA complexes in the absence of divalent metal ions were unsuccessful. As previously described for EcoRV (24), MunI (25), PvuII (26), and Cfr10I (27), a stable protein-DNA complex was formed in the presence of CaCl$_2$. The gel shift assay of wt Eco57I binding with the specific fragment in the presence of 10 mM CaCl$_2$ revealed a shifted DNA band at low (0.01 mM) protein concentration (Fig. 2). The amount of the initial complex increased with increasing protein concentration in the range of 0.01–0.1 mM and then progressively decreased as the protein concentration increased further (0.5–10 mM). Similar binding studies of wt Eco57I with the noncognate DNA revealed no shifted band corresponding to the initial complex. The wt Eco57I-DNA complexes in the presence of divalent metal ions were observed only at high protein concentrations (0.5–10 mM). Further, comparison of the wild-type Eco57I interaction with cognate and noncognate DNA indicates that the initial complex (Fig. 2) corresponds to the specific enzyme-DNA complex, whereas bands of lower mobility correspond to the complexes that are represented by DNA fragments bound both by specifically and nonspecifically interacting enzyme molecules (28). The binding pattern of the D78N and E92Q mutants to specific DNA fragments indicates that they retain the ability to generate specific complexes with cognate DNA similar to that of wt enzyme (Fig. 2). The same results were obtained with the D812A, D833N, and D833A mutant proteins (data not shown). At the same time, the single amino acid substitutions D78N and E92Q were likely to weaken nonspecific binding as judged by the absence (compared with wt Eco57I), of the clearly defined band with the nonspecific DNA fragment and the band of lowest mobility with the specific fragment at 10 mM protein concentration (see Fig. 2).

**Methylation Activity of Wild-type and Mutant Eco57I**—The cleavage-deficient mutants D78N and E92Q were also tested for methylation activity. The reactions were performed with purified proteins using the $\lambda$ DNA protection assay (see “Experimental Procedures”). Both mutants methylated DNA efficiently, and the specific activity of the D78N mutant was even higher than that of the wt Eco57I (Table I). The reaction mixture used in our experiments for assessing the cleavage activity of the wt and mutant Eco57I included AdoMet, an effective stimulator of endonucleolytic activity of the enzyme (4). It could not be excluded therefore that the observed cleavage deficiency of the D78N and/or E92Q mutants was attributed to the substitution of amino acids affecting the competition between cleavage and methylation activities of the bifunctional enzyme, rather than the amino acids involved in DNA cleavage catalysis per se. Such mutants in contrast to wt Eco57I, would initially methylate but not cleave DNA, rendering the modified DNA resistant to hydrolysis. AdoMet was therefore replaced by its analog Sinefungin, an inhibitor of the methylation reaction, in the reaction mixture to discriminate between these two alternatives: a cleavage deficiency as compared with preferential methylation mutant phenotypes. Sinefungin activated wt Eco57I as effectively as AdoMet (see Fig. 1). Under the same reaction conditions, no cleavage activity was detected in either the D78N or the E92Q mutant (Fig. 1).

**DISCUSSION**

**Eco57I Catalytic Site**—The results of mutational analysis in this study strongly suggest that of the two putative catalytic motifs $^{77}$PDX$_3$EAK (SM I) and $^{81}$PDX$_3$DQK (SM II), only the first one represents a DNA cleavage active site. Substitutions within SM I of negatively charged amino acids, which are most conserved in the catalytic centers of restriction endonucleases (8, 11), with functionally unrelated (D78A, D78K) or function-
ally similar (D78N, E92Q) amino acids reduced cleavage activity of the Eco57I to a level undetectable both in vivo and in vitro. Two mutants D78N and E92Q, which were selected for more detailed studies, however, retained the ability, as assessed by gel mobility shift assay, to interact with the target site specifically. The mutations also spared the DNA methylation activity of Eco57I restriction endonuclease, an observation which provides additional evidence that specific DNA binding as well as AdoMet binding were not affected by D78N and E92Q substitutions. Hence D78N and E92Q mutations display the properties expected for active site residue mutants; they uncouple the sequence-specific DNA binding and strand scission activities of the enzyme. However, bifunctionality of Eco57I suggested still another explanation for the observed phenotype of the mutant proteins. Namely, mutations that reverse the preferential order of expression of the two wild-type Eco57I enzymatic activities (cleavage then methylation) would be phenotypically indistinguishable from mutations affecting amino acid residues involved in catalysis/metal ion binding. This possibility was excluded by the observation that the endonucleolytic activity of the wt Eco57I was effectively stimulated not only by AdoMet but also by its analog Sinefungin. However, no DNA cleavage was detected regardless of which of the two cofactors was added to the reaction mixture with D78N and E92Q mutants. Some activity of the E92Q mutant (but not that of the D78N mutant) was detected only when the Mg$^{2+}$ in the standard reaction mixture was replaced with Mn$^{2+}$. Similar effects of metal ion replacement on enzymatic activities of the MunI catalytic mutants D83A and E98Q have been previously reported and explained on the basis of differential effects of the mutational replacements on the binding of Mg$^{2+}$ and Mn$^{2+}$ (25). This observation provides additional evidence to support the conclusion that the Asp-78 and Glu-92 residues of Eco57I are involved in catalysis and metal ion binding. The fact that the Asp-83 and Glu-98 residues of MunI (25) and the Asp-78 and Glu-92 residues of Eco57I occupy equivalent positions in the PDX,EXK motif of the respective enzymes is noteworthy.

Eco57I mutants containing single amino acid substitutions within SM II (D812A, D833N, and D833A) revealed only a small or moderate decrease of cleavage activity as compared with wt Eco57I to suggest that SM II does not represent the catalytic center. Circumstantial evidence supporting the conclusion that SM I represents the DNA cleavage center and SM II only mimics it as an amino acid sequence motif comes from an alignment of Eco57I and GsuI restriction endonucleases, whose recognition sequences are related (CTGAGG and CTGGAG, respectively). The homology of the enzymes is significant enough to conclude that they are evolutionary related (29). Of the two putative Eco57I catalytic motifs, only a homolog of SM I is represented in GsuI, whereas that of SM II is absent.

It has been demonstrated that as expected, an asymmetric DNA target is recognized by the FokI monomer (30), whereas cleavage of both strands of DNA is carried out by two FokI molecules dimerized through their endonucleolytic domains (14). The asymmetric nature of the Eco57I target sequence is also inconsistent with the use of a symmetric dimer for recognition and DNA cleavage. Whether a mechanism similar to that of FokI is used by Eco57I to cleave DNA remains to be determined.

Restriction endonucleases catalyze phosphodiester bond cleavage in double-stranded DNA in the presence of Mg$^{2+}$, leaving a 5′-phosphate and a 3′-hydroxyl group (8). The active sites of only a few of the numerous restriction endonucleases have been characterized so far (8). They share a triad of charged Asp, (Glu/Asp), and (Lys/Asp) amino acids. The catalytic relevance of the triad has been demonstrated not only for type II endonucleases but also for the type IIS restriction endonuclease FokI (13), another nontype II endonuclease BclI (31), and a few type I enzymes (32, 33). This study provides evidence that the catalytic/metal binding center of the type IV restriction endonuclease Eco57I is most likely also represented by a triad of charged amino acids Asp, Glu, and Lys located within the sequence motif 57I DNA cleavage region

100 aa

VIII, 558DKYFLFIREISQILKEGYGLYILPSRFI; VII, 594LRKFSENKYLQKL; VIII, 618SHQVFKNKT.

structure-function organization of Eco57I

The blocks of conserved amino acid residues common to γ group of DNA amino-methyltransferases are indicated and numbered. They were identified based on the similarity to the characterized methyltransferase motifs in Ref. 34. The conserved sequence motifs are: I, 263PTDPYVT; II, 253PDIACOSAPIVA; IV, 329PDIVGNPPYMATHEHMNOQ; V-VI, 599KYFLFIEISQILKEGYGLYILPSRFI; VII, 594LRKFSENKYLQKL; VIII, 618SHQVFKNKT.

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age activity by AdoMet, methylation of one DNA strand), substrate specificity (asymmetric recognition sequence), and cleavage mode (outside of the target duplex) of Eco57I, which are similar to the properties of type III restriction endonucleases. However, the following observations make the R-M fusion hypothesis unlikely. (i) In contrast to type III endonucleases, Eco57I is not stimulated by ATP (4); (ii) DEAD box motifs involved in ATP hydrolysis and DNA translocation that have been identified in Res subunit (37) are not present in the Eco57I amino acid sequence; and (iii) the size of the Eco57I endonuclease domain is ~350 amino acids (see Fig. 3), whereas that of Res subunits is 873–982 amino acids (38–40). Therefore generating the progenitor for the endonuclease domain of Eco57I from the Res subunit would require dramatic rearrangements of the latter.

The evolution of type IIS enzymes, which like Eco57I recognize asymmetric nucleotide sequences and cleave DNA at a defined distance from them but are not stimulated by AdoMet (neither contain the conserved motifs of MTases), possibly involves fusion of a DNA-specific-binding protein and an endonuclease (41). In type IIS R-M systems, two separate MTases exist, each specific for a different strand of asymmetric target duplex (42–44). The fusion of a single-strand-specific MTase with a DNA endonuclease could generate a progenitor of Eco57I, which potentially could reveal both DNA cleavage and methylation (of one strand). The cofactor requirement (Mg2+ and AdoMet), enzymatic activities (endonucleolytic, methylation of one DNA strand), and structure (a methylase fused with endonuclease) of such a hypothetical hybrid protein makes it a more likely candidate for the Eco57I predecessor than the product of Res-Mod fusion.

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