A Mutation in the Mod Subunit of EcoP15I Restriction Enzyme Converts the DNA Methyltransferase to a Site-specific Endonuclease*

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A closer inspection of the amino acid sequence of EcoP15I DNA methyltransferase revealed a region of similarity to the PDXₙ(D/E)XK catalytic site of type II restriction endonucleases, except for methionine in EcoP15I DNA methyltransferase instead of proline. Substitution of methionine at position 357 by proline converts EcoP15I DNA methyltransferase to a site-specific endonuclease. EcoP15I-M357P DNA methyltransferase specifically binds to the recognition sequence 5’-CAGCAG-3’ and cleaves DNA asymmetrically EcoP15I-M357P-DNA methyltransferase specifically binds to the recognition sequence 5’-CAGCAG(N)₁₀-3’, as indicated by the arrows, in presence of magnesium ions.

Sequence discrimination by DNA-binding proteins is one of the most remarkable examples of molecular recognition in biochemistry. Restriction-modification (R-M) systems are paradigms for the study of protein-DNA recognition. More than 3600 R-M enzymes have been identified so far, and they manifest 218 unique recognition specificities (1). These enzymes represent the largest family of functionally related enzymes. R-M systems comprise pairs of intracellular enzymatic activities: that is, restriction endonuclease (REase) activity and DNA methyltransferase (MTase) activity. The REase recognizes specific sequences and catalyzes cleavage of double-stranded DNA. The MTase prevents the REase from digesting endogenous DNA by modifying the same target sequence with the methyl group from 5′-adenosyl-l-methionine (AdoMet) (2). R-M enzymes are classified into types I, II, III, and IV, based on their recognition sequence, subunit composition, cleavage position, and cofactor requirements (3). The simplest R-M systems are the type II enzymes. These generally consist of two separate enzymes, one responsible for restriction and the other for modification of the cognate DNA sequences. The orthodox type II REases are homodimers that typically recognize a palindromic sequence of 4–8 bp and cut the DNA within or close to their recognition sequence in the presence of Mg²⁺ to give a 5′-phosphate and a 3′-hydroxyl end. Although thousands of type II REases are known, the structures of only 19 have been reported to date (4). Structural and mutational analysis of several type II REases reveal that the sequence PDXₙ(D/E)XK (n = 1–30 residues) acts as a catalytic/Mg²⁺ binding signature motif (5). For the great majority of type II REases, Mg²⁺ is an essential cofactor that can be substituted with Mn²⁺. The essential divalent ion cofactor is bound to the two carboxylates and the main carbon of the hydrophobic residue X of the PDXₙ(D/E)XK motif as well as to one of the non-bonding oxygens of the phosphate to be attacked. The fact that many REases have a common catalytic sequence motif suggests that they follow the same mechanism with at least one obvious exception, BfiI restriction endonuclease, which does not require a divalent metal ion cofactor (6).

Type III R-M systems are multisubunit and multifunctional enzymes that exhibit both restriction and modification activities. These enzymes are composed of two subunits, products of the res and mod genes, and require ATP, AdoMet, and Mg²⁺ for restriction. The Mod subunit alone functions as a methyltransferase in the presence of AdoMet and determines the specificity for methylation and restriction, whereas restriction activity requires the cooperation of both the Res and Mod subunits (7). DNA cleavage and ATP hydrolysis are functions of the Res subunit. The Res subunit lacks a DNA recognition function and is active only in conjunction with the Mod subunit.

Type III R-M enzymes characteristic recognize DNA sequences that lack symmetry and cleave 25–27 bp downstream of the sequence. Two inversely oriented (→ ←) unmethylated sites are the substrates for cleavage by type III restriction enzymes (8). In contrast, methylation proceeds regardless of the number and orientation of recognition sequences. It has been shown that ATP hydrolysis is required for DNA cleavage by the type III enzymes (9).

EcoP15I DNA methyltransferase (M.EcoP15I), a member of the type III R-M systems, adds a methyl group to the second adenine in the recognition sequence 5’-CAGCAG-3’ in the presence of AdoMet and magnesium (10). Using a Fenton chemistry affinity cleavage assay, we identified the magnesium binding-like motif as amino acids 355–377 (11). Sequence homology comparisons between EcoP15I DNA MTase and other restriction endonucleases allowed us to identify a PDXₙ(D/E)XK-like sequence as the putative magnesium ion...
Conversion of a Methyltransferase to an Endonuclease

binding site (Fig. 1). In M.EcoP15I, a PDX,(D/E)XK-like motif is present in which the partially conserved proline is replaced by methionine. Mutational analysis of this region and detailed biochemical characterization of mutant MTases are consistent with the fact that acidic amino acid residues of this region 355–377 in M.EcoP15I are important for the critical positioning of magnesium ions for catalysis. This is the first example of a metal-dependent function for a DNA methyltransferase (11). We have not found any MTases in the Protein Data Bank containing the Mg$^{2+}$ binding motif that is present in M.EcoP15I.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Wild type EcoP15I DNA MTase was expressed in Escherichia coli BL21(DE3)pLysS cells by transforming with plasmid pDN8, which harbors wild type M.EcoP15I. E. coli BL21(DE3)pLysS strain was used as a host for transformation of pET15b constructs carrying the mutant EcoP15I MTases.

E. coli strain ER2566 (a kind gift from New England Biolabs) was also used as host strain for transformation of mutant and wild type EcoP15I MTases. Plasmids pUC18 and pUC19 were used as substrates for methylation and cleavage assays. Plasmids pMDS32 (a kind gift from Dr. Mark D. Szczelkun, University of Bristol) and pUM1 were used as substrates in cleavage assays. E. coli strain DH5α was used as a host for preparing plasmids pUC18, pUC19, pMDS32, and pUM1. BL21(DE3)pLysS strain harboring pGEM3zf(–)M.EcoP15I-M357P was plated on LB agar with 100 µg/ml ampicillin, cells harboring pACY184 M.EcoP15I were plated on LB agar with 35 µg/ml chloramphenicol, and the double transformants were plated on LB agar with both ampicillin and chloramphenicol.

Overexpression and Purification of Wild Type and Mutant M.EcoP15I—Wild type EcoP15I DNA MTase was expressed and purified as described earlier (12). Mutant constructs (pET15b-M357P and pET15b-M357A) were transformed separately into E. coli BL21(DE3)pLysS cells. Cells harboring the mutant plasmids were grown at 37 °C in terrific broth (TB) containing 100 µg/ml ampicillin for 8 h (A$_{600}$ 1.0), IPTG (0.5 mM) added and grown for an additional 2 h. M.EcoP15I-M357P and M.EcoP15I-M357A were purified to near homogeneity as described earlier (12). The purity of the enzyme preparations were judged as being greater than 99% on SDS-PAGE. Protein concentration was estimated by the method of Bradford (13).

M.EcoP15I-M357P enzyme was expressed in this strain and purified as described above.

Site-directed Mutagenesis—Site-directed mutagenesis was done to introduce proline or alanine at position 357. The sequence of primer 1 (Table 1) was designed to change the methionine at position 357 to proline and create an SmaI restriction site. The sequence of primer 2 was designed to change the methionine 357 to alanine and to create a restriction site (PvuII). A single strand DNA template containing uracil residues was prepared for E. coli strain CJ 236 that harbored pGEM3zf(–)M.EcoP15I (11). Primers 1 and 2 were hybridized separately to the single-stranded DNA, and oligonucleotide-directed mutagenesis was performed as described previously (14). The resultant plasmids were termed pGEM3zf(–)M.EcoP15I-M357P and pGEM3zf(–)M.EcoP15I-M357A. The Ncol-BamHI fragment from these constructs carrying the mutant gene was cloned into Ncol-BamHI-digested pET15b. The mutants were identified by digesting the plasmid DNA with SmaI (M357P) or PvuII (M357A). The mutant genes were sequenced, and no other mutations except for the intended ones were found.

Similarly, site-directed mutagenesis of D355A, D358A, D370A, E372A, and E377A was carried out with primers 3–7 (Table 1) using pET15b-M357P as the template in separate PCR reactions. In this protocol, the entire lengths of both strands of the plasmid DNA are amplified in a linear fashion during several cycles of thermal cycling, generating a mutated plasmid containing staggered nicks on opposite strands (15). The constructs expressing the mutant proteins were initially screened by digestion with the corresponding restriction endonucleases (Table 1).

Preparation of $^3$H-Labeled pUC19 DNA—pUC19 plasmid was used to transform E. coli HB101, and the transformants were grown in M9 minimal medium containing 37 MBq/liter of [methyl-$^3$H]thymidine (Bhabha Atomic Research Centre, India). After continued growth at 37 °C overnight, cells were harvested by centrifugation, and a covalently closed circular form of the plasmid was purified as described (15). Analysis of the purified plasmid by electrophoresis through agarose showed that generally 95% of the DNA was the covalently closed circular form. DNA concentrations were assessed by measuring absorbance at 260 nm. Plasmid DNA preparation in which ~95% supercoiled form was used as substrate in cleavage reactions.

Methylation Assay—Methylation assays monitored incorporation of tritiated methyl groups into DNA by using a modified ion-exchange filter assay (16). Methylation assays were carried out in a reaction mixture containing 300 nm supercoiled pUC18 DNA, 1 µM [methyl-$^3$H]AdoMet in HEPES buffer (100 mM, pH 8.0) with wild type (1 µM), M.EcoP15I-M357P (1 µM), or M.EcoP15I-M357A (1 µM).

Electrophoretic Mobility Shift Assay—Increasing concentrations of wild type (0–5 µM) or mutant MTase (0–5 µM) were incubated with 100 nm 5’-end-labeled duplex I or II (Table 1) in binding buffer (50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 7 mM 2-mercaptoethanol, 10% (v/v) glycerol, and 1 mM EDTA) for 10 min on ice. Electrophoresis was performed as described (16). Gels were dried and exposed to x-ray film at room temperature for 16–20 h.
Conversion of a Methyltransferase to an Endonuclease

Photolabeling of Wild Type and Mutant EcoP15Is with [methyl-3H]AdoMet—Cross-linking wild type and mutant enzyme with AdoMet was done as described previously (17). M.EcoP15I (0.6 and 1.2 μM) or M.EcoP15I-M357P (0.6 and 1.2 μM) in reaction buffer (50 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 100 mM NaCl, 2 mM EDTA) were irradiated in the presence of 6.6 μM [methyl-3H]AdoMet (80 Ci/mmol). Photolabeled enzyme was visualized by exposing the dried gel to x-ray film at ~70 °C.

Gel Filtration Chromatography—Native molecular masses of wild type and M.EcoP15I-M357P were determined by gel filtration chromatography using a calibrated Superdex 200 column. The molecular masses of wild type and M.EcoP15I-M357P were calculated from the plot of V/v versus log molecular weight.

Cleavage Assays—DNA cleavage reactions with wild type and mutant enzymes were performed in HEPES buffer (100 mM, pH 8.0) containing 10 mM Mg2+ at 37 °C for 30 min (unless otherwise stated) in a reaction volume of 20 μl. DNA cleavage reactions with purified EcoP15I restriction endonuclease (R.EcoP15I) were performed at 37 °C for 30 min in the HEPES buffer in the presence of 1 mM ATP in a reaction volume of 20 μl. All other restriction digestions were performed with respective reaction conditions according to the manufacturer’s recommendations. Cleavage products were analyzed on 0.8% agarose gel electrophoresis.

Kinetic Methods—Steady-state kinetics were typically carried out by diluting the stock solution of M.EcoP15I-M357P enzyme to 20 nM and then adding a sample of the diluted enzyme to a solution of 3H-labeled pUC19 in buffer at 20 °C. Compositions of solutions given under “Results” and “Discussion” omit components from the enzyme dilution buffer. Samples were removed from the reaction mixture at timed intervals and immediately vortexed with 5 μl of stop mix (100 mM EDTA, 10 mM Tris, 60% (w/v) glycerol, and 0.03% μg/ml bromphenol blue, pH 8.0). Each sample was then analyzed by electrophoresis through 0.8% agarose gel containing 0.5 μg/ml ethidium bromide at 5 V/cm. Under these conditions the covalently closed circular form of pUC19 had a higher mobility than the open circle form of this DNA. For each sample from the reaction, slices of the gel that covered the covalently circular and linear forms of the DNA were excised, dissolved by incubation for 2 h at 67 °C in 0.5 ml of 5 M NaClO4, and added to 3 ml of water and 10 ml of scintillation fluid, and the radioactivity in each was determined by scintillation counting (18). Values for the amount of each form of the DNA were calculated as the percentage of the total DNA in that sample.

For steady-state kinetic experiments, zero time points were taken by adding stop mix to a sample of the DNA before the enzyme. For every reaction a sample from the same solution of DNA was also incubated with enzyme dilution buffer (but lacking enzyme) for the duration of the reaction. In all of the reactions described here, <1% of the covalently closed circular DNA was linearized in this control. Hence, the observed reactions must be due to M.EcoP15I-M357P enzyme rather than any contaminant nuclease that might have been present in the buffer. In a series of similar reactions containing M.EcoP15I-M357P (20 nM) and 3H-labeled pUC19 DNA, the concentration of DNA was varied in the range of 50–600 nM. An Eadie-Scatchard plot (v/[S] versus v) allowed the determination of $K_m^{DNA}$ and $V_{max}$. The turnover number ($k_{cat}$) was calculated as the ratio of $V_{max}$ to the enzyme concentration used. Data obtained were analyzed by regression analysis using Sigma Plot. The equations used to obtain the kinetic constants, $V_{max}^{DNA}$, $K_m^{DNA}$, and $k_{cat}$ were as described (19). Unless otherwise indicated, all enzyme activity data were the average of at least triplicate determinations.

Construction of Plasmid with No CAGCAG Sites (pLM1)—pMDS32, which has a single CAGCAG site, was used as a template to make a plasmid with no sites. The mutagenic primers used for changing CAGCAG to AAGCAG are listed in Table 1 (primers 8 and 9). The primers were designed such that an AflII site would be generated if the mutation occurred. Both the primers contain the desired mutation and have the same starting and ending position on opposite strands of the plasmid DNA. In this method the entire lengths of both strands of the plasmid DNA are amplified in a linear fashion during several cycles of thermal cycling, generating a mutated plasmid containing staggered nicks on opposite strands (15). The products of the PCR are treated with the restriction enzyme DpnI, which specifically cleaves fully methylated $^3$H-methylGATC sequences. DpnI will, therefore, digest the bacterially generated DNA used as a template for amplification. DpnI-resistant molecules, which are rich in desired mutants, are recovered by transforming DH5α cells with ampicillin as a selection marker. Plasmids with no CAGCAG sites were scored by AflIII digestion.

Mapping the Cleavage Site—Two primers were designed (primers 10 and 11, Table 1) for mapping the cleavage site. Although the forward primer (primer 10) sequence was 240 bp upstream, the reverse primer (primer 11) was 214 bp downstream of the CAGCAG site in pMDS32 DNA. Plasmid pMDS32 was cleaved by M.EcoP15I-M357P, and the reaction products were sequenced.

EcoP15I Methylated pUC18 DNA—pUC18 DNA was methylated by incubating with purified M.EcoP15I at 37 °C for 60 min in 100 mM HEPES buffer, pH 8.0, containing 0.25 mM EDTA, 6.4 mM MgCl2, 11 mM 2-mercaptoethanol, and 20 μM AdoMet. DNA was recovered by ethanol precipitation and used as a substrate in cleavage reactions.

RESULTS

Site-directed Mutagenesis, Expression, and Purification of Mutant EcoP15I MTases—Site-directed mutagenesis of Met-357 to Pro-357 or Ala-357 was carried out using primers 1 and 2 (Table 1) whose sequences were designed to change the methionine at position 357 to proline or alanine and create a Smal or a Pvull restriction site, respectively. Single-stranded DNA template containing uracil residues was prepared from E. coli strain C1236 that harbored pGEM3zf(−)M.EcoP15I. The mutant primers were hybridized separately to the single-stranded DNA, and oligonucleotide-directed mutagenesis was performed (14).

Similarly, site-directed mutagenesis of D355A, D358A, D370A, E372A, and E377A was carried out with primers 3–7 (Table 1) using pET15b-M357P as template in separate PCR reactions. The resultant plasmids were termed pGEM3zf(−)M.EcoP15I-M357P, pGEM3zf(−)M.EcoP15I-M357A, pET15b-M.EcoP15I-M357-D355A, pET15b-M.EcoP15I-M357-D358A, pET15b-M.EcoP15I-M357-D370A, pET15b-M.EcoP15I-M357-E372A, and pET15b-M.EcoP15I-M357-E377A.
Conversion of a Methyltransferase to an Endonuclease

**TABLE 1**

Oligonucleotides and duplexes used in this study

| Primer | Sequence | Amino acid changed | Restriction site created |
|--------|----------|--------------------|-------------------------|
| 1      | 5′-AAGCCTATGGGTATCCTCCGCCA-3′ | Met-357 to Pro | (+)/eliminated (−) |
| 2      | 5′-AAGCCTATGGGTATCCTCCGCCA-3′ | Met-357 to Ala | (+) |
| 3      | 5′-AAGCCTATGGGTATCCTCCGCCA-3′ | Asp-355 to Ala | (−) |
| 4      | 5′-CCATAGATGATGCTCGTATCTGTCG-3′ | Asp-358 to Ala | (−) |
| 5      | 5′-CTATTGATGATGCTCGTATCTGTCG-3′ | Asp-370 to Ala | (−) |
| 6      | 5′-CTATTGATGATGCTCGTATCTGTCG-3′ | Glu-372 to Ala | (−) |
| 7      | 5′-ATGTGATGATGCTCGTATCTGTCG-3′ | Glu-377 to Ala | (−) |
| 8      | 5′-ATGTGATGATGCTCGTATCTGTCG-3′ | Hgl (+) | (−) |
| 9      | 5′-GGTTAGTATGATGCTCGTATCTGTCG-3′ | AfIII (−) | (−) |
| 10     | 5′-GGTTAGTATGATGCTCGTATCTGTCG-3′ | AfIII (+) | (−) |

**FIGURE 2.** Toxicity of mutant M.EcoP15I. BL21(DE3)pLysS cells transformed with (A) pGEM3zf(−)M.EcoP15I (B) pGEM3zf(−)M.EcoP15I-M357P (C) pACYC184 M.EcoP15I or (D) pGEM3zf(−)M.EcoP15I-M357P and pACYC184 M.EcoP15I. Amp represents ampicillin, and Cm represents chloramphenicol.

pET15b-M.EcoP15I-M357P-D370A, pET15b-M.EcoP15I-M357P-E372A, and pET15b-M.EcoP15I-M357P-E377A resulted in the lysis of host cells BL21(DE3)pLysS (Fig. 2A). This toxicity was not observed when the host cell was transformed with pGEM3zf(−)M.EcoP15I (Fig. 2A). On the other hand, when host cells expressing wild type M.EcoP15I (pACYC184 M.EcoP15I) (Fig. 2C) was transformed with pGEM3zf(−)M.EcoP15I-M357P, double transfomers were seen to grow on LB plates containing both ampicillin and chloramphenicol (Fig. 2D).

**TABLE 2**

In vivo assay for putative endonuclease activity

| Plasmid constructs | Number of coloniesa |
|--------------------|---------------------|
|                    | −IPTG | +IPTG |
| M.EcoP15I (wild type) | 212 | 224 |
| M.EcoP15I-M357P | 136 | 15 |
| M.EcoP15I-M357P-D355A | 64 | 21 |
| M.EcoP15I-M357P-D370A | 107 | 32 |
| M.EcoP15I-M357P-E372A | 58 | 44 |
| M.EcoP15I-M357P-E377A | 168 | 149 |

a Values are average values determined from three independent experiments.

A 500-ml culture harboring pET15b-M357P or pET15b-M357A was grown for 8 h (A600 nm = 1.0), IPTG (0.5 mM) was added, and the culture was grown for an additional 2 h. At this point of time, cell lysis occurred. Using this as starting material, M.EcoP15I-M357P and M.EcoP15I-M357A were purified separately to homogeneity as described earlier (12). The purified M.EcoP15I-M357P enzyme was analyzed using SDS-PAGE and Western blotting (Fig. 3A) for alterations in electrophoretic mobility, and no apparent changes were detected vis-à-vis the wild type protein. Similarly, gel filtration analysis clearly showed that both the wild type and M.EcoP15I-M357P eluted as symmetric peaks at a position corresponding to a globular protein of ~150 kDa (Fig. 3B). It was observed that whereas the purified wild type M.EcoP15I was stable for at least 2 months when stored at 4 °C, the mutant M.EcoP15I-M357P lost all of its endonuclease activity within a week when stored under similar conditions.

**Methylation Activity and Substrate Binding Properties of M.EcoP15I-M357P—In vitro methylation assays** (16) that measured the ability to transfer 3H-labeled methyl group from [methyl-3H]AdoMet clearly showed that the mutant M.EcoP15I-M357P was inactive. Another mutant enzyme, where methionine at 357 was replaced by alanine (M.EcoP15I-
Conversion of a Methyltransferase to an Endonuclease

M57A), was catalytically active (Fig. 4A). We reasoned that the loss of activity of M.EcoP15I-M357P could be due to conformational changes in the protein structure or altered substrate binding properties. The effect of placing a proline at position 357 in EcoP15I DNA MTase was determined by measuring (for wild type and mutant enzymes) relative susceptibilities to proteolysis, UV, and circular dichroism spectroscopy. Gross conformational changes could not be detected between wild type and mutant enzymes (data not shown). To test if the ability of binding of substrates, DNA, and AdoMet were affected because of the substitution, binding properties of the wild type and mutant MTase were investigated. Using an electrophoretic mobility shift assay, both the wild type and mutant enzyme (M.EcoP15I-M357P) were shown to bind DNA almost with the same affinity (Fig. 4B). Exposure of wild type and M.EcoP15I-M357P to UV light in the presence of [methyl-3H]AdoMet results in strong photolabeling (Fig. 4C), suggesting that the mutant enzyme is still able to bind AdoMet.

DNA Cleavage Properties of M.EcoP15I-M357P—Incubation of M.EcoP15I-M357P with unmethylated supercoiled pUC18 DNA, which contains three EcoP15I recognition sites but with only one pair in the inverse orientation (→ ←), resulted in linearization of supercoiled pUC18 (Fig. 5A). On the other hand, incubation of pUC18 DNA with wild type M.EcoP15I or M.EcoP15I-M357A did not result in any cleavage (Fig. 5A, a and b). One could argue that the endonuclease-like activity could be due to a contaminating endonuclease, possibly the product of the endA1 gene present in the E. coli strain used. Therefore, we transformed the pET15b-M357P construct into E. coli strain ER2566. In this strain, the endA1 gene has been deleted. The mutant enzymes purified from this strain gave identical results to those described above (data not shown). When pUC18 DNA was premethylated by wild type EcoP15I MTase and used as a substrate in the above reaction, no linearization occurred (Fig. 5B, lane 3). This result clearly demonstrates that the M.EcoP15I-M357P recognizes unmethylated CAGCAG sequences and cuts DNA (Fig. 5B, lane 6). As a control, we performed digestion of unmethylated and methylated pUC18 DNA with the holoenzyme R.EcoP15I, which as stated earlier is composed of both the Mod and Res subunits. As can be seen clearly, the holoenzyme linearizes only unmethylated DNA (Fig. 5B, lane 5) and not methylated DNA (lane 2). When the holoenzyme was used, ATP was necessary, in the absence of which there was no cleavage (data not shown). The digestions with M.EcoP15I-M357P mutant enzyme were carried out only in the presence of Mg2+. We tested the effects of Mn2+, Ca2+, Co2+, and Zn2+, but none supported the DNA cleavage activity, although with Mn2+ there was cleavage but to a lesser extent (Fig. 5C). Only Mg2+ serves as a metal cofactor in the DNA cleavage reaction.

Recognition Site Requirement of M.EcoP15I-M357P—To unequivocally demonstrate that the mutant enzyme is not a nonspecific endonuclease, we constructed a plasmid pUM1, a derivative of pUC18 that does not contain any CAGCAG sequence (Fig. 6A). When this plasmid was used as a substrate in the DNA cleavage assays, the mutant enzyme or even the holoenzyme did not cleave the DNA (Fig. 6A, lanes 5 and 6, respectively). pUM1 has one AflII site, and when this plasmid was digested with AflII, supercoiled DNA was converted to a linear form, thus demonstrating that the plasmid DNA was cleavable (Fig. 6A, lane 4). A single mutation in the recognition sequence such as changing 5’-CAGCAG-3’ to 5’-AAGCAG-3’ abolished M357P REase activity (Fig. 6A). The specificity of the mutant endonuclease was, thus, established. Type III restriction enzymes R.EcoP15I and R.EcoP11 require two copies of the recognition sequence for efficient cleavage (20). It has been unequivocally demonstrated that covalently closed circular plasmid containing one EcoP15I recognition site is not a substrate for DNA cleavage by R.EcoP15I. On the other hand, we showed earlier that linear DNA molecules containing a single EcoP15I recognition site could be cleaved by R.EcoP15I (21). Therefore, we were interested to find out if M.EcoP15I-M357P mutant enzyme would cleave either of these two substrates. pMDS32 (22) is a pUC18 plasmid in which two of the three EcoP15I sites have been mutated such that it contains only one copy of the CAGCAG sequence (Fig. 6B). When supercoiled
plasmid pMDS32 was incubated with M.EcoP15I-M357P mutant enzyme in the absence of ATP but in the presence of Mg²⁺, the DNA was linearized (Fig. 6, lane 4). This result indicates that unlike the holoenzyme, R.EcoP15I, for the mutant M.EcoP15I-M357P enzyme, a single recognition site is sufficient for efficient cleavage.

The results described so far clearly demonstrate that supercoiled DNA containing one EcoP15I recognition site was linearized by M.EcoP15I-M357P. Even though pUC18 DNA used had more than one site, only linearization was observed. We, therefore, wondered if DNA topology had any influence on the cleavage by the mutant enzyme. The above experiments all used the covalently closed circular form of plasmid DNA directly as isolated from E. coli. The substrate would, therefore, be negatively supercoiled. The covalently closed circular (CCC) form of DNA could potentially affect the activity of the enzyme as compared with that an open circular (OC) form, because it can alter the conformation of the DNA.

Effect of DNA Topology on Cleavage Activity of M.EcoP15I-M357P—To test whether DNA topology affects the activity of the enzyme, pUC18 DNA was digested with two different type II restriction enzymes. The linear DNA molecules were then used as substrates. Interestingly, when HindIII- or AhdI-linearized pUC18 DNA was used as substrate in the DNA cleavage assay, no further cleavage was seen on incubation with M.EcoP15I-M357P (Fig. 7A, lanes 5 and 6). Increasing the
amounts of the mutant enzyme (5×) did not make any difference (data not shown). On the other hand, pUC18 DNA linearized previously with M.EcoP15I-M357P was further cleaved by the type II restriction enzyme, BamHI (Fig. 7B, lane 4). When nicked circular pUC18 DNA was incubated with the mutant enzyme, no change in the mobility of the DNA could be seen (Fig. 7A, compare lanes 7 and 8). These observations indicate that the mutant M.EcoP15I-M357P enzyme cleaves only supercoiled DNA but does not act on nicked or linearized DNA. It, therefore, appears that the rate and/or the mode of DNA cleavage by the enzyme varies with the topological state of the DNA. This possibly indicates that the interaction of the enzyme with its recognition site may involve unwinding of the DNA. Alternately, the possibility of a contamination with topoisomerase, which also possesses a DNA cleavage activity, in our enzyme preparations cannot be
Conversion of a Methyltransferase to an Endonuclease

ruled out. M.EcoP15I-M357P enzyme cleaves DNA in the absence of ATP, and therefore, a topoisomerase II kind of activity is ruled out. Inclusion of classical inhibitors of topoisomerase II like ciprofloxacin or novobiocin in our assays have no effect on the cleavage pattern (data not shown). Importantly, we do not observe any topoisomerase I-like activity in our enzyme preparations, and more importantly, incubation of covalently closed circular pUC19 DNA with topoisomerase I did not seem to affect the cleavage pattern (data not shown). Additionally, we have performed DNA cleavage experiments with the mutant enzyme and pUC19 DNA in the absence and presence of a single-strand oligonucleotide containing the EcoP15I recognition sequence 5'-CAGCAG-3' and oligonucleotide without this sequence. In all cases we obtained the same result i.e. there was no significant difference in the cleavage activity in the absence or presence of these oligonucleotides. If supercoiling were partially unwinding the recognition site, one might expect a high concentration of the unwound sequence to interfere with the hydrolysis. The results presented here indicate that substrate topology influences the behavior of M.EcoP15I-M357P. At present, we do not understand the details of the topology requirement. It has been shown that the ability of BspMI to detect the relative orientation of two DNA sequences depends on both the topology and the length of the intervening DNA (23) and, thus, can act as a sensor of the conformational dynamics of supercoiled DNA. Cleavage of CCC, OC, or linear DNA by M.Ecp15I-M357P could not be stimulated in trans by the addition of oligonucleotides containing the EcoP15I recognition sequence when concentrations varied from 0.5- to 5-fold excess (data not shown).

Steady-state Cleavage by M.EcoP15I-M357P—Reactions were carried out under steady-state conditions with the enzyme at a much lower concentration than the DNA. Under these conditions the cleavage of all the plasmid requires multiple enzyme turnovers, each involving its binding to a DNA molecule, its catalytic reactions, and its subsequent dissociation from the product (s) before attacking another molecule of DNA.

Cleavage of ³H-labeled pUC19 plasmid DNA substrate containing three recognition sites was first studied in multiple-turnover kinetic experiments with limiting enzyme (20 nM) and an excess of substrate (200 nM). Samples were withdrawn from the reactions at increasing time points and analyzed by agarose gel electrophoresis. The amounts of radioactive DNA substrate and product at each time point were quantified. Analysis of time course of DNA hydrolysis by M.EcoP15I-M357P at pH 8.0, 10 mM MgCl₂, and 20 °C revealed a significant increase in linear DNA product over the first minute of the reaction (Fig. 8A) followed by a slower steady-state phase. Reaction rates were calculated from either the decrease in the concentration of the substrate (covalently closed circular DNA) with time or the corresponding increases in the amount of DNA product (linear DNA). Clearly, the enzyme functions catalytically rather than stoichiometrically. Such a reaction profile is consistent with product burst kinetics. From these analyses, it appears that the enzyme cleaves the substrate without yielding any open circular DNA and instead progressed directly to the linear form (Fig. 8A, inset). The enzyme, thus, cleaves both DNA strands at a single recognition site at rates greater than its dissociation from the cleaved DNA. This profile is consistent with a concerted mechanism in which the enzyme cuts both strands of the DNA dur-
ing the lifetime of a single enzyme-DNA complex. Reaction profiles for type II restriction enzymes differ considerably among these enzymes, from the concerted cleavage of both strands of DNA through to separate and independent reactions at each strand (4). Furthermore, as can be seen from Fig. 8B, the rate of DNA cleavage was initially linear with increasing concentrations of DNA and later saturated around 200 nM. The velocities followed a hyperbolic curve against the concentration of pUC19 DNA. Fitting these data to the equation for a rectangular hyperbola gave the kinetic constants. The lines drawn are the best-fits to the Michaelis-Menten equation. From the Eadie-Scatchard plot (Fig. 8B, inset) the $K_m$ for DNA was calculated to be $2.3 \times 10^{-7}$ M, and the $k_{cat}$ was determined to be 7 min$^{-1}$. Next, cleavage of plasmid DNA substrates containing one, two, and three recognition sites was studied in multiple turnover kinetic experiments with limiting enzyme (57 nM) and excess of substrate (120 nM) (Fig. 8C). Qualitatively, it appears that the enzyme cleaves the three substrates in similar manner, although there is some preference to the substrate containing two sites. As before, the reaction of the enzyme on any of the three substrates yielded none of the open circular form of DNA but progressed directly to the linear form (data not shown).

**Determination of Cleavage Sites of M.EcoP15I-M357P**—The holoenzyme R.EcoP15I (Res$_2$ Mod$_2$) recognizes a 5'-CAG-CAG-3' sequence and cuts 25–27 bp downstream to the 3'-end of the recognition site only in the presence of ATP and AdoMet (7). The mutant enzyme described here is a dimer of Mod subunits, Mod$_2$, and cleaves DNA in the absence of ATP or AdoMet. We were interested to find out if the cleavage by the mutant enzyme occurred 25–27 bp downstream to the 3'-end of the CAPGAG sequence. We performed DNA cleavage using the one site plasmid, pMDS32. The linearized DNA fragment was sequenced using two specific primers 10 and 11 (Table 1). The cleavage site was mapped to be 10 nucleotides downstream of the recognition site in the upper strand and right at the start of the recognition site in the lower strand (Fig. 9). Clearly, the mutant enzyme and the holoenzyme recognize the same recognition sequence, 5'-CAGACAG-3', but cleave at different distances away from the recognition site.

A 16-base pair staggered break seems very striking, but it is not uncommon when one analyzes breaks generated by DNA-
cleaving enzymes. For instance, restriction products of EcoBl, a type I R-M enzyme, have much longer terminal deletions, suggesting the formation of long 3′ overhangs. Formation of 3′-overhangs up to 100 nt long have been described for EcoBl (24).

**DISCUSSION**

The majority of type II restriction enzymes are homodimers that typically recognize symmetric sequences and cleave the DNA within or immediately adjacent to their recognition site and do not depend on binding to a second copy of their recognition site for maximum activity (4). The asymmetric nature of the EcoP15I recognition sequence is inconsistent with the use of a symmetric dimer for recognition and DNA cleavage, as found for the type I restriction enzymes. A single enzyme 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 as was demonstrated with FokI, a type IIS enzyme (25). These enzymes are generally monomers that transiently associate to form dimers to cleave both DNA strands. Their reactions involve bridging interactions between two copies of their recognition sequence. The dimerization triggers a conformational change, which leads to double-strand cleavage (26). Because M.EcoP15I-M357P exists as a dimer in solution (Fig. 3B), it may interact with DNA in a manner different from that of FokI. M.EcoP15I-M357P cleaves its one-site substrate directly to linear DNA without liberating the nicked form of the DNA. Having cut one site in its two-site substrate, M.EcoP15I-M357P, does not seem to cleave the residual site. It is also clear from Fig. 7 that M.EcoP15I-M357P is unable to cleave linear DNA. In this respect, the reaction of M.EcoP15I-M357P on pUC19 deviates from the behavior expected for a type II restriction enzyme on a substrate with three recognition sites. With M.EcoP15I-M357P, it is, therefore, possible that the active sites from the two subunits might be juxtaposed at the subunit interface to create a structural unit capable of inflicting a double-strand break in DNA. In terms of its mode of action, M.EcoP15I-M357P is like a type IIS enzyme (26) but recognizes an asymmetric DNA sequence. The enzyme-DNA complex must, therefore, be organized differently from other type IIS enzymes. To identify how the mutant enzyme operates, its active-site organization and its oligomeric status when bound to DNA needs to be determined.

For a long time type II REases were considered to be unrelated in evolution. Only after the determination of the crystal structures of a few of these enzymes did it become clear that there was a structural similarity between different and seemingly unrelated enzymes (4). Because of divergent evolution, different subtypes exist with differences in quaternary structure, domain organization, and active site architecture on the one side and mechanistic details on the other side. Since 1995, when crystal structures of restriction endonucleases became available, it was realized that these proteins do have a similar structural core that harbors the active site with the characteristic PD... (D/E)XXK motif (27, 28). It is now clear that type II restriction enzymes of the PD... (D/E)XXK developed from a common ancestor with divergent evolution. It is certainly surprising that the PD... (D/E)XXK-like motif is found in the EcoP15I DNA methyltransferase. Fuxreiter and Simon (29) analyzed the crystal structures of the PD... (D/E)XXK superfAMILY in relation to protein structure stability elements and found a high correlation between the active site residues and the stabilization factors that contribute to preventing structural decay. This motif is not only characteristic of the majority of type II restriction enzymes but is also found among types I, III, and IV restriction enzymes as well as other nucleases. It is becoming clear that the arrangement of charged groups in restriction enzyme active sites can result in unusual and complex behavior. For instance, examination of the amino acid sequence of Nael, a type IIE restriction enzyme, uncovered similarity to the active site of human DNA ligase I, with leucine 43 in Nael instead of the lysine essential for ligase activity. Nael, a novel DNA endonuclease, shows topoisomerase and recombinase activities when the lysine residue is substituted for leucine 43 (30). The cryptic endonuclease activity in EcoP15I DNA MTase perhaps provides an evolutionary link between restriction endonucleases and the methyltransferase protein families. Our results clearly indicate that structure-function relationships in restriction enzymes active sites can be complex and that the ensemble of conserved charged residues, which mediate DNA hydrolysis in Mg2+-dependent nucleases, constitutes a critical link between function and conformation (31).

In summary, the engineered M357P EcoP15I MTase is an active, sequence-dependent restriction endonuclease that cuts away from its recognition sequence only in the presence of Mg2+. Unlike the holoenzyme R.EcoP15I, the engineered endonuclease does not require AdoMet or ATP for DNA cleavage and does not require two sites in the inverted orientation. It is of potential interest to use such an engineered enzyme as a genetic manipulation tool.

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Conversion of a Methyltransferase to an Endonuclease

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