Probing the Role of Cysteine Residues in the EcoP15I DNA Methyltransferase*

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Chemical modification using thiol-directed agents and site-directed mutagenesis has been used to investigate the role of cysteine residues of EcoP15I DNA methyltransferase. Irreversible inhibition of enzymatic activity was provoked by chemical modification of the enzyme by N-ethylmaleimide and iodoacetamide. 5,5′-Dithiobis(2-nitrobenzoic acid) titration of the enzyme under nondenaturing and denaturing conditions confirmed the presence of six cysteine residues without any disulfides in the protein. Aware that relatively bulky reagents inactivate the methyltransferase by directly occluding the substrate-binding site or by locking the methyltransferase in an inactive conformation, we used site-directed mutagenesis to sequentially replace each of the six cysteines in the protein at positions 30, 213, 344, 454, 553, and 577. All the resultant mutant methylases except for the C344S and C344A enzymes retained significant activity as assessed by in vivo and in vitro assays. The effects of the substitutions on the function of EcoP15I DNA methyltransferase were investigated by substrate binding assays, activity measurements, and steady-state kinetic analysis of catalysis. Our results clearly indicate that the cysteines at positions other than 344 are not essential for activity. In contrast, the C344A enzyme showed a marked loss of enzymatic activity. More importantly, whereas the inactive C344A mutant enzyme bound S-adenosyl-l-methionine, it failed to bind to DNA. Furthermore, in double and triple mutants where two or three cysteine residues were replaced by serine, all such mutants in which the cysteine at position 344 was changed, were inactive. Taken together, these results convincingly demonstrate that the Cys-344 is necessary for enzyme activity and indicate an essential role for it in DNA binding.

EcoP15I DNA methyltransferase (EcoP15I DNA MTase)

catalyzes the transfer of a methyl group from S-adenosyl-l-methionine (AdoMet) to the second adenine nucleotide in the canonical site 5′-CAGCAG-3′ (1) to form N⁵-methyladenine. The enzyme is part of the type III restriction-modification (R-M) system (2). Type III R-M enzymes are multifunctional proteins that exert both methylation and restriction activities.

(2). Type III R-M systems contain two subunits, the Res subunit encoded by the res gene and the Mod subunit encoded by the mod gene. Although the Mod subunit alone can catalyze the methylation reaction, both the Res and Mod subunits are necessary for DNA cleavage. The enzymes have an absolute requirement for ATP for restriction, and recently we and others (3, 4) showed that ATP hydrolysis was required for DNA cleavage. It has been shown that only the Mod subunit is involved in DNA sequence recognition in both the restriction and modification reactions (5). We had earlier shown by gel mobility shift assays that EcoP15I DNA MTase binds about 3-fold more tightly to DNA containing its recognition sequence 5′-CAGCAG-3′ than to nonspecific sequences in the absence or presence of cofactors. Interestingly, in the presence of ATP, the discrimination between specific and nonspecific sequences increased significantly (6, 7).

Based on the type of methylation catalyzed and amino acid sequence analysis, DNA MTases are divided into three classes (8). m4C-MTases are enzymes that methylate the exocyclic amino group of cytosine to form N⁴-methylcytosine, and m6A-MTases methylate the exocyclic amino group of adenine to form N⁶-methyladenine. The third class contains the m5C-MTases, that methylate cytosine residues at the C-5 position to form C⁵-methylcytosine. Comparative analyses have shown that m5C-MTases share an ordered set of sequence motifs that alternate with non-conserved regions (9–13). Among the well conserved motifs, motif I (FXGFXG) can be seen in all three classes of DNA MTases as well as in protein and RNA MTases. All methyltransferases utilize AdoMet as methyl donor, and it was proposed that motif I is involved in AdoMet binding (9, 14). The tertiary structures of the HhaI and HaeIII DNA MTases (belonging to m5C-MTases) and TaqI DNA MTase (member of the N6A-MTase) bound to AdoMet (15–17) clearly indicate that motif I forms a part of the AdoMet binding pocket.

Structural analysis has found striking similarity between DNA MTases of the two classes, namely the m5C- and m6A-MTases. This suggested that many AdoMet-dependent MTases may share a common catalytic domain structure. Guided by this common catalytic domain structure, a multiple sequence alignment of 33 m6A- and 9 m4C-MTases revealed that these two classes of MTases were more closely related to one another and to the m5C-MTases than was expected (18). Based on this analysis, m4C- and m6A-MTases do not group separately from one another. The amino MTases belong to three groups distinguished by differences in the linear orders of conserved motifs in their primary sequences. The three groups are named α, β, and γ (18). To date only two DNA amino MTases have been structurally characterized, the group γ N6mA MTase M.TaqI (17) and the group β N4mA MTase, M.PvuII (19).

EcoP15I DNA MTase as mentioned earlier, is an N⁶-adenine MTase and belongs to the β group of amino MTases. We have recently demonstrated that altering amino acid residues in the motif I of EcoP15I DNA MTase resulted in loss of AdoMet
binding but left DNA target recognition unaltered (20). A second motif characteristic of m6A-MTases and m4C-MTases, (N/D/S) PP (Y/F) (motif IV) (21), is well conserved in EcoP15I DNA MTase. Substituting tyrosine in motif IV of EcoP15I DNA MTase by site-directed mutagenesis resulted in loss of enzyme activity although we observed enhanced cross-linking of AdoMet and DNA. These results reinforce the importance of motif IV in catalysis (20).

Cysteine residues are particularly important for studying the structure and function of enzymes. In m5C-MTases, motif IV consisting of amino acids FPCQ has been shown to be the catalytic center. The invariant Pro-Cys dipeptide is known to be involved in methyl group transfer (22–24). In the catalytic mechanism of m5C-MTases, it has been shown that the cysteine residue of motif IV and C-6 of the target cytosine form a covalent intermediate during the methyl group transfer from AdoMet (22). Mutation of this cysteine in many Cs-MTases abolishes the enzyme activity without affecting DNA recognition and cofactor binding (23–25). Although it has been firmly established that the thiol in the only conserved cysteine among m5C-MTases that carries out the attack at C-6 of cytosine and is important for the methylation reaction (22, 25), very little is known about the roles of cysteine residue(s) in any N6A-Mtase-catalyzed reaction. Rubin and Modrich (26) demonstrated that the thiol in the only conserved cysteine among m5C-MTases is important for the methylation reaction (22, 25), very little is known about the roles of cysteine residue(s) in any N6A-Mtase-catalyzed reaction. Rubin and Modrich (26) demonstrated that EcoRI MTase rapidly lost activity upon cysteine modification by exposure to N-ethylmaleimide (NEM). Everett et al. (27) showed that NEM modification of cysteine 223 in EcoRI MTase was responsible for the loss of enzyme activity.

Initial experiments done in our laboratory suggested that the absence of reducing agents in buffers used during purification of EcoP15I DNA MTase resulted in loss of enzyme activity; when purified enzyme preparations were dialyzed against buffers not containing any reducing agent, the enzyme lost activity on storage. These results suggested that cysteine residues in the protein could have a role in stabilization or in catalysis. EcoP15I DNA MTase, a 645-amino acid protein, contains six cysteine residues at positions 30, 213, 344, 434, 553, and 577 as deduced from the DNA sequence of the mod gene (28) (Fig. 1). By using oligonucleotide-directed site-specific mutagenesis, each of the six cysteines were substituted with another amino acid, and the mutant enzymes were purified and characterized. As a part of an investigation to study structure-function relationships in this enzyme, we were interested in assessing the role(s) of cysteine residues, if any, in DNA recognition, cofactor binding, or in catalysis.

In the present investigation we have used chemical modification and site-directed mutagenesis studies to elucidate the role of the cysteines in the activity of EcoP15I DNA MTase. Our findings indicate that cysteine at position 344 is required for enzyme activity.

**EXPERIMENTAL PROCEDURES**

*Materials—[methyl-3H]AdoMet (80 Ci/mmol) was purchased from Amersham Corp., United Kingdom. Iodoacetamide (sodium salt), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and N-ethylmaleimide (NEM) were purchased from Sigma. Centricon 30 microconcentrator units were purchased from Amicon. [α-32P]dATP (3000 Ci/mmol) and [γ-32P]ATP (3500 Ci/mmol) were purchased from Bhabha Atomic Research Center, Bombay, India. T4 polynucleotide kinase, Klenow DNA polymerase, T4 DNA ligase, and all restriction enzymes were obtained from New England Biolabs. Restriction enzymes AccI and Ncol and DNA sequencing kit were obtained from U. S. Biochemical Corp. Sep-Pak cartridges (Millipore) were a gift from Dr. H. A. Vasavada. PD-10 gel filtration columns were purchased from Pharmacia, Sweden. EcoP15I restriction enzyme (R. EcoP15I) was purified from Escherichia coli cells harboring a pBR322-based plasmid containing the res-mod genes of p15I, a resident plasmid of E. coli 15 T. All other chemicals used in this study have been described elsewhere (6, 20).

**Bacterial Strains and Plasmid Vectors—**E. coli JM109 was used as a transformation host for plasmid pDN8 carrying M. EcoP15I gene under lambda phage P1 promoter (29). E. coli B strain BL21(DE3) with a phage lysogene (innn 21 int) that contains the phage T7 RNA polymerase gene under the lac UV5 promoter was used as a host for propagating the plasmid pGEM3zf(−) M. EcoP15I-C344A and C344S. Plasmid pDN8 was used for overexpression of wild-type M. EcoP15I (29). The EcoRI-HindIII fragment from pDN8, carrying the entire M. EcoP15I gene, was subcloned into plasmid pGEM3zf(−). This construct is referred to as pGEM3zf(−) M. EcoP15I (20). E. coli CJ236 (det * ung *) was used as host for preparation of single-stranded DNA templates for mutagenesis. JM109 was used as a host for transformation of plasmid constructs derived from pUC18 as well as for overexpression and purification of mutant methylases. JM109 cells harboring the plasmid pSH1182 (carrying the gene for EcoP15I restriction enzyme) were used for *in vivo* restriction assays.

**General Recombinant Techniques—**Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were purchased and used according to the manufacturers’ recommendations. Digestions with type II restriction enzymes, ligations, transformations and DNA electrophoresis were done as described by Sambrook *et al.* (30). Plasmid DNA (pUC18, pUC19, pBR322, or pGEM3zf(−)) was prepared as described by Sambrook *et al.* (30).

**Oligodeoxyribonucleotides and Radiolabeling—**The following oligonucleotides were used in the mutagenesis reactions as primers: primer A (21-mer), d(TCTCTCGTCGTTAACTAGTTT); primer B (21-mer), d(GATCTCGTCGTTAACTAGTTT); primer C (21-mer), d(GATCTCGTCGTTAACTAGTTT); primer D (24-mer), d(GAGTGTGTTGTGTTCACTGCTTCT); primer E (24-mer), d(AAGGTTGTTGTGTTGAAGGCTTCT); primer F (27-mer), d(TGATTTCTCCTCGTTGCTGTTGTTGTTGGA); primer G (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer H (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer I (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer J (21-mer), d(TACTACAGGGACCATGTAGT); primer K (30-mer), d(CAAATACAGTCGACCATGCTCTCCATCCGATGCAAAAGAT); primer L (21-mer), d(GCTCTTCAATCGGATCAAGGTTTATTCTT); primer M (21-mer), d(AATCAGGACCATGTAGT); primer N (30-mer), d(CAAATACAGTCGACCATGCTCTCCATCCGATGCAAAAGAT); primer O (21-mer), d(AATCAGGACCATGTAGT); primer P (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer Q (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer R (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer S (21-mer), d(GCTCTTCAATCGGATCAAGGTTTATTCTT); primer T (21-mer), d(AATCAGGACCATGTAGT); primer U (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer V (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer W (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer X (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer Y (21-mer), d(CCTTCCATCAATGCGAAAAGA); primer Z (21-mer), d(CCTTCCATCAATGCGAAAAGA). The underlined region of the oligonucleotide represents the EcoP15I recognition sequence. Basic procedures for labeling of oligonucleotides were performed as described (6).

**Construction of Cysteine Substitution Mutants and Purification of Mutant EcoP15I DNA MTases—**Site-directed mutagenesis was done to replace the cysteine residues at positions 30, 213, 344, 434, 553, and 577 by serine, asparagine or serine or tryptophan, alanine or serine, or tyrosine or alanine, and serine and serine, respectively, using suitable primers A to K. The sequence of primer A was designed to change cysteine at position 30 to serine. Single-stranded DNA template containing uracil residues was made from E. coli strain CJ236 that harb-
bored pGEM3Zf(−) M. EcoP15I. Primer A was hybridized to this single-stranded DNA, and oligonucleotide-directed mutagenesis was performed essentially according to the method of Kunkel (31). The resultant plasmid was termed pGEM3Zf(−) M. EcoP15I-C30S. The mutants were then identified by dideoxy chain termination sequencing.

The sequence of primer D was designed to change cysteine 344 to tryptophan and to create a restriction site EcoRV with a mismatch in the plasmid DNA with primer D. The resultant plasmid was termed pGEM3Zf(−) M. EcoP15I-C213SN. The mutants were done using single-stranded DNA from pGEM3Zf(−) M. EcoP15I-C213SN as a template and primer E. The resultant plasmid was termed pGEM3Zf(−) M. EcoP15I-C213S lost the HpaI site, and therefore mutants could be easily screened. The sequence of primer D was designed to change cysteine 344 to tryptophan and to create a restriction site EcoRV with a mismatch in the plasmid DNA with primer D. The resultant plasmid was termed pGEM3Zf(−) M. EcoP15I-C344Y, and mutants were scored by digesting the plasmid DNA with Ncol. Two separate rounds of mutagenesis were done using single-stranded DNA from pGEM3Zf(−) M. EcoP15I-C344Y as a template and primers E and F. The resultant plasmids were termed pGEM3Zf(−) M. EcoP15I-C344W and oligonucleotide-directed mutagenesis was performed as described earlier using primer D. The resultant plasmid was termed pGEM3Zf(−) M. EcoP15I-C344W, and mutants were scored by digesting the plasmid DNA with Ncol. Two separate rounds of mutagenesis were done using single-stranded DNA from pGEM3Zf(−) M. EcoP15I-C344W as a template and primers E and F. The resultant plasmids were termed pGEM3Zf(−) M. EcoP15I-C344S and pGEM3Zf(−) M. EcoP15I-C344A lost the HpaI site, and therefore mutants could be easily screened. The sequence of primer D was designed to change cysteine at position 553 to threonine. The resultant plasmids were termed pGEM3Zf(−) M. EcoP15I-C553S and pGEM3Zf(−) M. EcoP15I-C553T. All the mutants were identified by dideoxy chain termination sequencing. The double mutants were constructed using single-stranded DNA from plasmid pGEM3Zf(−) M. EcoP15I-C213SN as a template. Mutagenesis reactions were carried out using two primers in each case. In five separate reaction mixtures the reactions primers C and D were used in these were primer A, E, H, I, and J, respectively. As mentioned earlier, primer C was designed such that the asparagine at position 213 was changed to serine, and in addition the HpaI restriction site would be lost. The second pair of primers used in the five mutagenesis reactions were designed to change the cysteine residues at positions 30, 434, 435, 553, and 577 to serine and either introduced a new restriction site or resulted in the removal of the restriction site. The resultant plasmids were termed pGEM3Zf(−) M. EcoP15I-C213S/C30S, pGEM3Zf(−) M. EcoP15I-C213S/C344S, pGEM3Zf(−) M. EcoP15I-C213S/C344S, pGEM3Zf(−) M. EcoP15I-C213S/C535S, and pGEM3Zf(−) M. EcoP15I-C213S/C577S. In order to construct M. EcoP15I with three mutations (triple mutants), the following scheme was employed. To create M. EcoP15I-C30S/C213S/C344S, the EcoRI-EcoRV fragment (see Fig. 1) of pGEM3Zf(−) M. EcoP15I-C30S/C213S was ligated with EcoRI-EcoRV-digested pGEM3Zf(−) M. EcoP15I-C344S DNA. In order to construct clones expressing M. EcoP15I-C30S/C213S/C344S and M. EcoP15I-C30S/C213S/C535S and pGEM3Zf(−) M. EcoP15I-C577S, the EcoRV-HindIII fragments of pGEM3Zf(−) M. EcoP15I-C30S/C213S/C535S and pGEM3Zf(−) M. EcoP15I-C577S were separately cloned into EcoRV-HindIII sites of pGEM3Zf(−) M. EcoP15I-C30S/C213S. All mutations were confirmed by dideoxy chain termination sequencing, and the entire mutant mod genes were sequenced by automated DNA sequencing method.

DNA fragments containing the individual mutations were released from the respective pGEM3Zf(−) constructs (excluding the double and triple mutants) using suitable restriction sites on either side of the mutations (Fig. 1), and these fragments were separately swapped into the EcoRI-EcoRV-digested pGEM3Zf(−) M. EcoP15I DNA restriction sites of the resultant plasmids, for instance pC30S, pC213N, pC344S, etc., were used for expression and purification of mutant EcoP15I DNA MTases. pGEM3Zf(−) M. EcoP15I-C344A and pGEM3Zf(−) M. EcoP15I-C344S were used to purify C344A and C344S mutant enzymes.

Overexpression and Purification of Wild-type and Mutant EcoP15I DNA Methyltransferases—Wild-type and mutant EcoP15I DNA methyltransferases were purified according to the method of Rao et al. (29) to near homogeneity. The hosts from which the wild-type and all cysteine mutants, except C344A and C344S, were purified were the same. A different host had to be used for the purification of C344A and C344S mutant enzymes. Plasmids pGEM3Zf(−) M. EcoP15I-C344A and pGEM3Zf(−) M. EcoP15I-C344S were transformed into BL21(DE3) cells. Cells were grown to an A600 of 0.7 and then induced by adding isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.5 mm. The cells were harvested after incubation for 3 h.

Sulfonylhydrazide Modification—Aliquots (1 ml) of EcoP15I DNA MTase were dialyzed overnight (16 h) at 4 °C against nonreducing buffer (10 mm sodium phosphate buffer, pH 7.0, 100 mm NaCl, 0.1 mm EDTA and 10% glycerol). Unless otherwise mentioned, all experiments were conducted in nonreducing buffer. Following exchange into nonreducing buffer, the MTase concentration was determined using a Bradford protein assay (32), standardized with known amounts of MTase. Dialyzed wild-type EcoP15I DNA MTase was incubated each with DTNB (5 mm), NEM (5 mm), and iodacetamide (5 mm) at room temperature for 30, 5, and 30 min, respectively, and the percent activity remaining was measured. NEM was dissolved in absolute alcohol and kept at −20 °C as a stock solution (200 mm). A given amount of the purified dialyzed enzyme was incubated at 25 °C for 5 min with various amounts of NEM in 10 mm potassium phosphate buffer (pH 7.0) containing 1 mm EDTA. After incubation, the reaction mixture was transferred to the methylation reaction buffer, and the enzyme activity was measured. These experiments have been performed three times using different enzyme preparations. The variation in the values was in the range of 3–5%.

Determination of Sulphydryl Groups—The amount of free sulphydryl group in the wild-type M. EcoP15I was determined spectrophotometrically at 412 nm using 5,5'-dithiobis(2-nitrobenzoic acid) (33). The enzyme (final concentration 1.3 μm subunits) was incubated at 25 °C with 12.1 mM DTNB in 0.1 mM potassium phosphate buffer (pH 7.0). In separate experiments under the same assay conditions, reaction with DTNB was followed more directly by continuous monitoring of absorbance at 412 nm over 3 h. The stoichiometry of the reaction was calculated by using the extinction coefficient of 14,150 M−1 cm−1 for the thionitrobenzoate (TNB2−) anion in the absence of any denaturant and a value of 13,700 M−1 cm−1 in the presence of guanidinium chloride or SDS. In yet another experiment, wild-type EcoP15I DNA MTase was incubated with either 6 mM guanidinium chloride or 1% SDS for 4 h at 40 °C, and DTNB titration was carried out as described above, and the change in absorbance at 412 nm was monitored. In order to determine the number of unreacted NEM, and the residual enzyme activity was determined. These experiments have been performed three times using different enzyme preparations. The variation in the values was in the range of 3–5%.

In Vivo Restriction Assay—Modification in vivo by mutant MTases was assessed by the effectiveness with which they protected lambda (λ) phage from the EcoP15I restriction-modification system. The efficiency of plating (EOP) of these phages on a r− strain (JM109 cells) reflects the level of in vivo methylation.

Sensitivity to Restriction Endonuclease—Plasmid DNAs, carrying wild-type or mutant MTases were isolated using the alkaline lysis method (34) and then digested with EcoP15I restriction enzyme. Typically 1.0 μg of plasmid DNA was digested with purified EcoP15I restriction enzyme for 60 min at 37 °C followed by Proteinase K(500 ng) treatment at 56 °C for 60 min. The digestion products were analyzed by 0.8% (w/v) agarose gel electrophoresis in the presence of ethidium bromide.

In Vivo Methylation Activity—MTase activity was monitored by incorporation of tritiated methyl groups into pUC18 DNA, and the specific activity of the enzyme was measured as described (6). All assays were repeated at least three times. Initial rate data were fitted by nonlinear regression to the Michaelis-Menten equation. Steady-state kinetic parameters (Km and Vmax) of EcoP15I transferase were determined using saturating concentrations of pUC18 DNA. Reaction rates were determined at different AdoMet concentrations. Analysis of kinetic data was done using methods described (29). The enzyme concentrations refer to the amount of subunits based on a molecular mass of 75 kDa.

Circular Dichroism Measurements—Circular dichroism (CD) measurements were taken using a Jasco J20C spectropolarimeter. All experiments were repeated at least three times. Initial rate data were fitted by non-linear regression to the Michaelis-Menten equation.
ments were done at 25 °C in 20 mm potassium phosphate buffer (pH 7.0). The protein solutions were incubated for 10 min in 1-mm path length quartz cells in a final volume of 400 μl prior to recording the CD spectrum at the wavelengths indicated. The protein samples were dialyzed extensively against 20 mM potassium phosphate (pH 7.0) before recording the measurements. The observed ellipticities were converted to mean residue ellipticity [θ]_{222}/l, by using Equation 1 (50).

\[
[\theta]_{222} = \frac{[\theta]_{222}/l}{100} \text{ (Eq. 1)}
\]

where [θ]_{222} is the observed ellipticity in degrees; mrw is the mean residue molecular weight based on a molecular mass of 75 kDa and 645 amino acids; c is the protein concentration in grams/ml, and l is the path length of the cell in centimeters.

**Photolabeling of Wild-type and Mutant EcoP15I DNA MTases with {methyl}-H\textsubscript{2}AdoMet—AdoMet cross-linking was done as described (35).** Electrophoretic Mobility Shift Analysis—Binding reactions were performed in 50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10 mM MgCl\textsubscript{2}, 7 mM 2-mercaptoethanol, 10% (v/v) glycerol, and 1 mM EDTA. Typical reactions of 10 μl were incubated for 10 min on ice and loaded onto a 6% polyacrylamide gel. Electrophoresis was performed as described (6). The gels were dried on Whatman 3MM paper and subjected to autoradiography to Kodak XAR film.

**Chemical Cross-linking of Wild-type and Mutant EcoP15I DNA MTases—**Cross-linking reactions of the proteins with glutaraldehyde were carried out by incubating the enzymes (2 μg) in 0.1 M phosphate buffer (pH 8.0) containing 0.25 mM EDTA. Glutaraldehyde was added to a final concentration of 0.1% to the above mixture. Cross-linking was carried out at 4 °C for 5 min. Reactions were stopped by adding SDS-loading dye and boiling the samples for 2 min. The reactions were analyzed on a 2.5–8% gradient polyacrylamide gel containing 0.1% SDS. The gel was silver-stained to visualize the protein bands.

**Immunoblotting—**Polyclonal antibodies to the denatured wild-type EcoP15I DNA MTase were raised in a rabbit. For Western blot analyses, E. coli lysates or the purified recombinant enzyme were subjected to polyacrylamide gel electrophoresis (PAGE) after solubilization with 6M guanidinium chloride. SDS-PAGE analysis was essentially the same results. SDS-PAGE analysis in the absence or presence of 2-mercaptoethanol revealed no differences in the electrophoretic mobility of the wild-type enzyme (data not shown). It is clear that under nondenaturing conditions, four of the six cysteines in the protein reacted with DTNB (Table I). In the presence of 6 M guanidinium chloride, the remaining two cysteines react with DTNB (Table I). These results clearly indicate the possible absence of disulfides in the enzyme and corroborate the amino acid sequence data of the protein which shows the presence of six cysteine residues (28). To examine whether disulfide bonds were present in M. EcoP15I and, if present, were important for activity, we tested the effects of DTT, a strong disulfide bond reducing agent. It was found that the enzyme did not lose more than 2% activity when incubated for 5 h with 10 mM DTT (data not shown). Treatment of the enzyme with DTT had no effect on the electrophoretic mobility of the protein clearly indicating the absence of disulfide bonds. These experiments were carried out at least three times using different enzyme preparations with essentially the same results. SDS-PAGE analysis in the absence or presence of 2-mercaptoethanol revealed no differences in the electrophoretic mobility of the wild-type enzyme (data not shown). These results suggest that EcoP15I DNA MTase contains no disulfide bonds. Treatment of the DTNB-treated enzyme with DTT (100 mM) to remove the thiol-modifying reagent restored the enzyme activity (>80%), indicating that the changes caused by DTNB were reversible (data not shown).

To study the effect of NEM on the activity of EcoP15I DNA MTase, the enzyme was first dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 10% glycerol, and 1 mM sodium EDTA. This is because NEM is known to modify lysine residues at pH greater than 8.0, although the reaction is very slow. The absence of 2-mercaptoethanol in this buffer resulted in inactivation of the enzyme upon storage. As mentioned earlier, for M. EcoP15I, it was necessary to add 2-mercaptoethanol to all buffers to stabilize the enzyme during purification and storage (29). Inactivation kinetics were carried out with freshly dialyzed enzyme at 0.5, 1.0, 2.0, and 3 mM NEM. The modification reaction was arrested by the addition of

| Treatment | No. of –SH groups/mmol of protein |
|-----------|---------------------------------|
| None      | 3.6                             |
| SDS (5%)  | 15.2                            |
| SDS (5%) + DTT (100 mM) | 4.9                      |
| GdnHCl (6 mM) | 5.6                       |
| GdnHCl (6 mM) + DTT (100 mM) | 5.2                      |

**RESULTS**

**Effect of Thiol Reagents on EcoP15I DNA Methytransferase Activity—**Incubation of purified EcoP15I DNA MTase with thiol reagents N-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) inactivated the enzyme. More than 95% activity was inhibited by these reagents. Incubation of the enzyme with iodoacetamide (5 mM) failed to significantly inhibit the activity. However, higher concentrations (100 mM) of iodoacetamide did result in loss of activity (data not shown). These results demonstrate that sulfhydryl groups in EcoP15I DNA MTase may be necessary for enzyme activity.

**Titration of Wild-type EcoP15I DNA MTase with DTNB—**The free sulfhydryl content of EcoP15I DNA MTase was quantified, initially without denaturation, with the objective of confirming that the cysteines were exposed on the surface of the protein. A stock solution of the purified EcoP15I DNA MTase (78 μM) was placed in a 3-m1 spectrophotometer cell and kept at 25 °C. To this solution, 100 μl of 20 mM DTNB solution was added and mixed rapidly. The concentration of DTNB (625 μM) in the reaction mixture was 80.1-fold excess of the total concentration of sulfhydryl groups (7.8 μM sulfhydryl group). The concentration of TNB\textsuperscript{2−}, which is released when DTNB reacts with sulfhydryl groups, increased rapidly in the first 30 min and then remained almost constant through 60 min. From the concentration of TNB\textsuperscript{2−} released for 150 min, the number of sulfhydryl groups titrated with DTNB was calculated (Table I). About 4 mol of DTNB reacted with 1 mol of subunit of the EcoP15I DNA MTase; that is among six sulfhydryl groups present in a subunit, only four sulfhydryl groups reacted with DTNB. EcoP15I DNA MTase activity rapidly decreased for the first 30 min and then was completely lost at 60 min by the reaction with DTNB (data not shown).

The total number of thiols present in the enzyme was quantified by reaction with DTNB under nonreducing conditions in the absence and presence of 6 M guanidinium chloride or 1% SDS at pH 7.0. It is clear that under non-denaturing conditions, four of the six cysteines in the protein reacted with DTNB (Table I). In the presence of 6 M guanidinium chloride, the remaining two cysteines react with DTNB (Table I). These results clearly indicate the possible absence of disulfides in the enzyme and corroborate the amino acid sequence data of the protein which shows the presence of six cysteine residues (28). To examine whether disulfide bonds were present in M. EcoP15I and, if present, were important for activity, we tested the effects of DTT, a strong disulfide bond reducing agent. It was found that the enzyme did not lose more than 2% activity when incubated for 5 h with 10 mM DTT (data not shown). Treatment of the enzyme with DTT had no effect on the electrophoretic mobility of the protein clearly indicating the absence of disulfide bonds. These experiments were carried out at least three times using different enzyme preparations with essentially the same results. SDS-PAGE analysis in the absence or presence of 2-mercaptoethanol revealed no differences in the electrophoretic mobility of the wild-type enzyme (data not shown). These results suggest that EcoP15I DNA MTase contains no disulfide bonds. Treatment of the DTNB-treated enzyme with DTT (100 mM) to remove the thiol-modifying reagent restored the enzyme activity (>80%), indicating that the changes caused by DTNB were reversible (data not shown).

**NEM Modification of EcoP15I DNA Methytransferase—**Of the available sulfhydryl reagents, N-ethylmaleimide has consistently been used for cysteine modification because of its high selectivity for –SH groups. The reaction with NEM involves the nucleophilic attack upon its olefinic bond by the reactive sulfhydryl (–SH) group of cysteine in the active site of the enzyme. This leads to the formation of a covalent adduct. To study the effect of NEM on the activity of EcoP15I DNA MTase, the enzyme was first dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 10% glycerol, and 1 mM sodium EDTA. This is because NEM is known to modify lysine residues at pH greater than 8.0, although the reaction is very slow. The absence of 2-mercaptoethanol in this buffer resulted in inactivation of the enzyme upon storage. As mentioned earlier, for M. EcoP15I, it was necessary to add 2-mercaptoethanol to all buffers to stabilize the enzyme during purification and storage (29). Inactivation kinetics were carried out with freshly dialyzed enzyme at 0.5, 1.0, 2.0, and 3 mM NEM. The modification reaction was arrested by the addition of

| Treatment | No. of –SH groups/mmol of protein |
|-----------|---------------------------------|
| None      | 3.6                             |
| SDS (5%)  | 15.2                            |
| SDS (5%) + DTT (100 mM) | 4.9                      |
| GdnHCl (6 mM) | 5.6                       |
| GdnHCl (6 mM) + DTT (100 mM) | 5.2                      |
an excess of 2-mercaptoethanol. The inactivation curves show that only concentrations as high as 3 mM NEM brought about significant inactivation. This suggested that probably a slow reacting cysteine residue was involved in catalysis. The linear plots of the logarithm of residual enzyme activity against the reaction time indicate that the time-dependent decrease in activity displayed first-order kinetics (Fig. 2A). The apparent first-order rate constants \( K_{\text{app}} \) were calculated from the slopes of the inactivation plot for each concentration of NEM used. B. pseudo first-order plot. The apparent first-order rate constants \( K_{\text{app}} \) were plotted against log[NEM]. The y intercept gives the rate of inactivation of the enzyme \( K_{\text{inact}} \). The slope of this line gives the number of cysteine residues modified (n). Values are averages of triplicate determinations.

![Image](66x360 to 280x729)

**FIG. 2**. Kinetics of inactivation of M. EcoP15I by N-ethylmaleimide. M. EcoP15I (0.4 mg/ml) was incubated at 30 °C in 50 mM sodium phosphate (pH 7.0) containing 0–3 mM NEM. At the indicated times, aliquots were withdrawn and assayed for DNA MTase activity as described under “Experimental Procedures.” A. time course of inactivation plot was constructed (not shown). Control incubations gave no change in activity. A. time course of inactivation. The pseudo first-order rate constants \( K_{\text{app}} \) were calculated from the slopes of the inactivation plot for each concentration of NEM used. B. pseudo first-order plot. The apparent first-order rate constants \( K_{\text{app}} \) were plotted against log[NEM]. The y intercept gives the rate of inactivation of the enzyme \( K_{\text{inact}} \). The slope of this line gives the number of cysteine residues modified (n). Values are averages of triplicate determinations.

definition of the modification of a cysteine residue involved in methyl group transfer or because of modification at the substrate-binding sites. To investigate the latter possibility, the enzyme was incubated with AdoMet or DNA, prior to modification with NEM. In both cases, there was no significant protection offered by the substrates against NEM inactivation (data not shown). Substrate protection was also investigated through a binding assay as described under “Experimental Procedures.” For AdoMet cross-linking experiments, the formation of a stable adduct was first demonstrated for the MTase dialyzed against phosphate buffer devoid of 2-mercaptoethanol (Fig. 3A, lane 2). When this enzyme was treated with 5 mM NEM prior to cross-linking, there was a drastic decrease in the intensity of the adduct formed (Fig. 3A, lane 4). However, upon preincubation of the enzyme with the radioactive substrate, adduct formation was partially restored (Fig. 3A, lane 3). To test whether NEM modification eliminates M. EcoP15I binding to its recognition sequence, we tested the inactivated protein for its ability to bind an oligonucleotide duplex (duplex I) containing the EcoP15I recognition sequence. Gel mobility shift tests were done to assess the functional ability of the M. EcoP15I that had been preincubated with NEM binds to form a complex with DNA just like the untreated or mock-treated enzyme which produced a retarded complex (Fig. 3B).

Circular dichroism spectra (200–250 nm) were collected for both the native and the NEM-modified enzyme and were used to calculate the secondary structure of the enzyme (Fig. 4A). The data suggested that the secondary structure of the enzyme was not significantly perturbed by NEM modification. The oligomeric nature of the NEM-modified enzyme was determined by glutaraldehyde cross-linking. As can be seen from Fig. 4B, treatment with glutaraldehyde of both the unmodified and NEM-modified enzyme clearly indicates the dimeric nature of these proteins.

**Activities of Mutant EcoP15I DNA Methyltransferases—**Two tests were done to assess the functional ability of the M.
EcoP15I mutants to modify DNA. First, modification in vivo by mutant MTases was assessed by the effectiveness with which they protected non-modified lambda phages from restriction by EcoP15I R-M system. Cells harboring pGEM3Zf(−)-derivative plasmids (described above) were infected with phage λvir, at a titer high enough (10^6 plaque-forming units/ml) to give confluent lysis on plating. Phage lysate was prepared from these plates by standard protocol (30). The titer of the resulting lysate was determined on an r m strain. The EOP of these phages was calculated as the ratio of plaque-forming units/ml obtained in an r m strain to r m strain. It is evident from Fig. 5A that an EOP of 0.8 was obtained in case of wild-type MTase, indicating almost complete protection against restriction. Although five mutants (C30S, C213S, C434S, C553S, and C577S) had an EOP value similar to the wild-type, four mutants (C30S, C213S, C553S, and C577S) had an EOP value similar to the wild type, four mutant MTases using glutaraldehyde. The enzymes (2–4 μg) were cross-linked with 0.1% glutaraldehyde and reactions analyzed as described under “Experimental Procedures.” Lane 1, NEM-modified M. EcoP15I and glutaraldehyde; lane 2, wild-type M. EcoP15I and glutaraldehyde; lane 3, wild-type enzyme alone; and lane 4, molecular weight markers. + and − indicate treatment of M. EcoP15I with and without NEM.

EcoP15I restriction enzyme was determined in vitro by R. EcoP15I. Fig. 6 shows that the plasmid DNA from cells expressing the mutant M. EcoP15I-C213S was not digested by R. EcoP15I, whereas plasmid DNA from cells expressing M. EcoP15I-C213S, M. EcoP15I-C344A, and M. EcoP15I-C344S was not protected. Similarly, results obtained with the double and triple mutants were confirmed using the in vitro assay. It is quite clear from Fig. 7 that any plasmid DNA that encoded mutant mod genes in which the cysteine at position 344 was changed was suscep-
cross-linking experiments were performed. When purified M. EcoP15I was incubated at 4 °C with [methyl-3H]AdoMet and then subjected to short wavelength UV irradiation for 60 min, the enzyme was labeled as detected by SDS-PAGE followed by fluorography and autoradiography (Fig. 9). Exposure of purified mutant MTases to UV light in the presence of labeled AdoMet resulted in cross-linking of radioactivity to these enzymes including the C344A enzyme.

**Kinetic Properties of the Mutant EcoP15I DNA Mtases**—To compare the catalytic capabilities of mutant and wild-type EcoP15I DNA MTases more fully, some kinetic parameters were evaluated for the enzymes. At saturating DNA concentrations, linear primary plots (data not shown) were obtained for variation of the AdoMet concentration (eight concentrations over a 20-fold range) for both the wild-type and the mutant enzymes. Initial velocities were plotted against AdoMet concentration, from which \( V_{\text{max}} \) and \( K_m \) were determined. There was no evidence of nonlinearity in the dependence of the activity on pUC18 DNA concentration and thus no suggestion that the three methylation sites on this substrate were not equivalent. It is possible that differences between rates of methylation of, or binding to, each of the three sites might be too subtle to be detected by a kinetic analysis. Specific activity values for wild-type and mutant enzymes were in the range of 45–60 pmol of methyl group transferred per min/mg. The substitution of cysteines at positions 30, 213, 434, 553, and 577 by serine in the EcoP15I DNA MTase did not significantly affect the \( V_{\text{max}} \) value. The Michaelis constants (\( K_m \)) for AdoMet were not significantly altered, suggesting that the mutant enzymes were able to bind AdoMet almost as efficiently as the wild-type enzyme. When compared with the wild-type, the C213S enzyme displayed an AdoMet concentration that gave a \( K_m \) about half that of wild type. Although the \( k_{\text{cat}} \) values for all mutant enzymes were similar to the wild-type enzyme, the C30S had a value one-fifth of the wild-type (Table II). The other parameter generally used to compare engineered mutant enzymes is the "specificity constant," \( k_{\text{cat}}/K_m \). From Table II, it is clear that the specificity constant for AdoMet is the same in the case of all mutant enzymes except the C30S mutant enzyme which has a 5-fold lower value.

**Physical Behavior of EcoP15I DNA Methyltransferase Mutants**—Cysteine residues are known to affect protein folding and conformational stability due to the formation of disulfide bonds. SDS-PAGE analysis of mutant MTases revealed that they all behave indistinguishably from wild-type EcoP15I DNA MTase (Fig. 8A). We had earlier reported that the wild-type enzyme exists as a dimer of molecular mass 150,000 Da in solution (6, 7). We determined the oligomeric nature of the mutant enzymes by employing glutaraldehyde cross-linking of the subunits. The purified mutant methylases exist as the same molecular species as the wild-type enzyme, as is evident from glutaraldehyde cross-linking of the proteins (data not shown). All purified mutant MTases were subjected to PAGE under nondenaturing conditions without prior treatment with reducing agent. All of them behaved exactly as the wild-type enzyme suggesting that the oligomeric nature of the enzymes did not change as a result of amino acid substitution (data not shown). During purification each of the cysteine mutants, except C344S mutant MTase, behaved indistinguishably from wild-type EcoP15I DNA MTase (data not shown).

**Characterization of EcoP15I-C344A Mutant Enzyme**—Several attempts were made to purify EcoP15I-C344A and EcoP15I-C344S mutant enzymes using the same strategy that was employed to purify the other mutant MTases. However, each time the proteins rapidly degraded either during the
dialysis step or during chromatography on DEAE-Sephacel matrix. We therefore expressed these mutant genes in another host background, *E. coli* B strain BL21(DE3) instead of JM109. The former is a *lon* strain, and we therefore assumed that proteolysis could be minimized. However, we could not purify the C344S mutant enzyme because the protein degraded during the dialysis step. As can be seen from Fig. 10A, the C344A mutant enzyme preparation appeared to be substantially pure (about 70% homogeneous). Western blot analysis (Fig. 10B) confirmed the presence of the enzyme protein in this preparation. This enzyme preparation did not show any significant methylation activity as compared with the wild-type enzyme (Fig. 10C). In order to find out if loss of activity was due to loss of AdoMet or DNA binding, we performed UV cross-linking of AdoMet and the C344A mutant enzyme and studied its DNA binding properties. It is evident from Fig. 11A that the extent of UV cross-linking of AdoMet to wild-type and mutant C344A enzyme was similar (compare lanes 1 and 3). Interestingly, there was very little difference in AdoMet cross-linking, when NEM-treated C344A mutant enzyme was used (Fig. 11A, compare lanes 3 and 4). On the other hand, when NEM-treated wild-type enzyme was used, there was no significant cross-linking (Fig. 11A, compare lanes 1 and 2). The C344A mutant enzyme failed to bind to DNA containing the *EcoP15I* recognition sequence (Fig. 11B, compare lanes 3 and 6). In order to find out if replacement of Cys-344 might have led to a structural abnormality in the MTase, limited proteolysis was used to probe the accessibility of glutamic acid residues to V8 protease in the wild-type and C344A enzymes. The results gave no indication of a major structural change; both enzymes displayed essentially identical rates and patterns of degradation when incubated at a V8 protease:protein ratio of 1:100 for 0–30 min (data not shown).

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**Fig. 8.** SDS-polyacrylamide gel electrophoresis and immunoblotting of purified wild-type (WT) and mutant *EcoP15I* DNA MTases. Electrophoresis and immunoblotting were performed as described under “Experimental Procedures.” A, SDS-polyacrylamide gel electrophoresis. Proteins in the gel were stained with Coomassie Blue. B, immunoblotting. Protein samples were denatured in the presence of 2-mercaptoethanol. The transfer blot was immunostained with polyclonal antibodies against M. *EcoP15I*.

**Fig. 9.** Characterization of *EcoP15I*-C344A mutant enzyme. A, SDS-PAGE analysis of partially purified M. *EcoP15I*-C344A. Lane 1, standard molecular mass markers; lane 2, 3 μg of enzyme preparation. B, immunoblot. 3 μg of the enzyme preparation was run on 0.1% (w/v) SDS-8% (w/v) polyacrylamide gel. Protein was detected by Western blotting with rabbit antiserum against *EcoP15I* Mod subunit (1–10,000 dilution). C, activity of wild-type and C344A mutant enzyme. The assays were done as described under “Experimental Procedures.”

**Table II**

| Enzyme     | \( K_m \) (μM) | \( k_{cat} \) (min\(^{-1}\) × 10\(^{-3}\)) | \( k_{cat}/K_m \) | Relative \( h_{cat}/K_m \) |
|------------|----------------|---------------------------------|------------------|---------------------------|
| Wild type  | 0.83           | 5.8                             | 6.98             | 1.0                       |
| C30S       | 0.76           | 1.09                            | 1.43             | 0.2                       |
| C213S      | 0.47           | 4.0                             | 8.51             | 1.2                       |
| C553S      | 0.71           | 4.63                            | 6.52             | 0.93                      |
| C577S      | 0.73           | 4.96                            | 6.79             | 0.97                      |

**Fig. 10.** Kinetic parameters of wild-type and mutant *EcoP15I* DNA MTases. *EcoP15I* DNA MTase activity was measured as described under “Experimental Procedures.” All assays were performed at 37 °C.

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Fig. 11. A, AdoMet cross-linking. Lane 1, wild-type enzyme; lane 2, wild-type enzyme plus NEM; lane 3, C344A enzyme; and lane 4, C344A enzyme and NEM. Arrow indicates the position of the adduct. B, DNA binding assay. Lane 1 and 4, no protein; lane 2, wild-type enzyme; lane 3, wild-type enzyme and sinefungin; lane 5, C344A mutant enzyme; and lane 6, C344A mutant enzyme and sinefungin.

**DISCUSSION**

Cysteines can serve as specific points for covalent labeling by radioactive, fluorescent, and spin-labeled -SH-reacting compounds because of their reactivity. These can then be used to probe three-dimensional structures and to detect intramolecular conformational changes. With this aim in mind, the current study combines results from protein chemistry and mutagenesis in order to elucidate the role of cysteine residues in the EcoP15I DNA methyltransferase. Whereas preincubation of M. EcoP15I DNA MTase with DTNB resulted in the loss of enzymatic activity, addition of DTT to DTNB-labeled EcoP15I DNA MTase under native conditions removed about 90% of the attached probe with concomitant recovery of MTase activity. Treatment of the enzyme with DTT showed that no disulfides were essential to maintain its activity. These observations clearly demonstrated that covalent modification of a cysteine residue was directly responsible for the observed inactivation.

In the present work, the involvement of cysteine residues in the catalytic function of EcoP15I DNA MTase was also indicated by the inactivation of the enzyme by the thiol-specific reagent NEM. Kinetic analysis of the reaction of NEM demonstrates that modification of only 1 cysteine/single subunit resulted in loss of activity (Fig. 2). This comprehensive inhibition with a range of thiol reagents of different size and complexity argues strongly in favor of a significant structural or functional role for cysteine residues in EcoP15I DNA MTase. Inactivation could be caused by the steric obstruction of the active site of the enzyme, conformational alterations of the enzyme, or modification of a cysteine residue essential for the catalytic process. It is possible that the introduction of the large hydrophobic N-ethylsuccinimidyl group of NEM and not the loss of the sulfhydryl group was the cause of the inactivation that we observed. Our results clearly show that although the NEM-modified enzyme binds specifically to DNA (Fig. 3B), AdoMet binding was significantly decreased (Fig. 3A). There are a number of instances where it has been shown that the modification or oxidation of cysteine residues in DNA-binding proteins affects the ability of these proteins to bind DNA and therefore enzymatic activity. For instance, Aiken et al. (39) have shown that NEM inactivated RsrI endonuclease by producing a modified enzyme that was unable to bind its recognition sequence. The sensitivity of T4 DNA-[N\(^{35}\)]-adenine methyltransferase (40), M. Eco Dam (41), and M. EcoRI (27) to NEM indicated that one or more cysteine residues was important for activity. It was observed that M.BspRI, a C5-Mtase, was able to accept the methyl group from AdoMet in the absence of DNA. Self-methylation was, however, inhibited by sulfhydryl reagents, and two cysteines were identified that bind the methyl group in form of S-methylcysteine (42).

Chemical modification studies on EcoP15I DNA MTase using thiol reagents suggest that cysteine(s) is the likely target of this reaction. Modification results in a fast inactivation process even though cysteine(s) may not be involved in the catalytic mechanism. Inactivation of an enzyme as a result of modification accompanied by protection against inactivation by competitive inhibitors or substrates for the enzyme is generally used as a criterion to assess whether modification is active site-directed. We were unable to do substrate protection experiments because at high concentrations of AdoMet, M. EcoP15I exhibits substrate inhibition (29). We therefore carried out site-directed mutagenesis to define more precisely the role of the six cysteine residues in substrate binding and catalysis. It has been observed in some instances that replacement of cysteine by site-directed mutagenesis led to conclusions different from those reached by chemical modification studies, especially when a bulky thiol reagent such as NEM was used (43). This turned out to be true in our case (see below).

Although cysteine and serine are chemically similar in the sense that they are both nucleophilic, they possess significant differences in nucleophilicity and polarity. The hydroxyl group of serine is highly polar, and the sulfhydryl group of cysteine is relatively non-polar. Assuming that cysteine occupies a position in a hydrophobic environment, the introduction of a polar residue like serine could significantly perturb the active enzyme structure. In order to address this possibility, a second mutation was generated that replaced cysteine at position 344 by the non-polar residue alanine. Mutation to alanine was chosen as this is the most conservative change in terms of size and charge, and any resulting functional change can be largely attributed to the loss of the thiol group. The six cysteine residues of EcoP15I DNA MTase located in positions 30, 213, 344, 434, 553, and 577 were each individually and in combination mutated to serine. All substitutions except at position 344 resulted in an active phenotype as assessed both in *in vivo* and *in vitro* assays (Figs. 5–7). Replacement of cysteine at position 344 either with serine or alanine resulted in an inactive enzyme. This therefore suggested that all other cysteines were relatively benign. As in the case of the C344S mutant, the C344A mutant enzyme was inactive clearly indicating the importance of cysteine at position 344 in maintaining the activity of EcoP15I DNA MTase. Replacing the thiol of Cys-344 by hydrogen (C344A) or substitution of a hydroxyl group for the thiol (C344S) had a drastic effect, resulting in loss of activity. These observations therefore suggest that EcoP15I DNA MTase appears to be sensitive to the change in polarity and/or differences in hydrogen bonding properties caused by the substitution of serine or alanine residue at position 344. Gabbara et al. (44) have shown that replacement of the catalytic cysteine in EcoRII methyltransferase, a m5C-MTase, by serine resulted in a mutant enzyme with a catalytic efficiency about 10,000 times less than that of wild-type.

NEM inhibited each of the purified mutant enzymes except the C344A enzyme in the same manner as the unaltered enzyme, demonstrating that these altered proteins contain a residue modifiable by NEM. The observation that the NEM-treated C344A mutant enzyme behaves identically to the NEM-modified wild-type enzyme in terms of AdoMet binding convincingly demonstrates that Cys-344 is modified by NEM. Although the activity of C344A mutant enzyme was almost negligible (Fig. 10C), it can be argued that the insignificant amount of activity could be possible due to occasional misreading of serine codon by a cysteynil tRNA or could be the result of
contamination by the wild-type enzyme during purification. This possibility can be eliminated by the fact that the C344A mutant enzyme behaves almost identically to the wild-type when both are subjected to NEM modification followed by AdoMet cross-linking (Fig. 11A). C344A mutant enzyme could be catalytically inactive for a variety of reasons, including those associated with conformational changes. If AdoMet or DNA did not bind, or if the dimeric nature was not maintained, an inactive enzyme would be formed. It is clear from Fig. 11 that the C344A mutant enzyme binds to AdoMet but not to DNA, and therefore the loss of activity is due to the inability of the enzyme to bind one of its substrates. The observation that the C344A mutant enzyme was unable to bind DNA (Fig. 11) is in contrast to the observation that the NEM-modified enzyme was able to bind DNA (Fig. 3). However, these two contrasting effects can be explained if we assume that cysteine at position 344 is crucial for DNA-MTase interaction. Modification of this residue did not alter this interaction, but replacement of this residue with alanine abolished the interaction. The hydrogen bonding capabilities of the side chains of cysteine and alanine are different, and therefore substitution of alanine in the presence of cysteine affects DNA-MTase interactions. The dimeric nature of the mutant enzyme was not altered as is evident from both limited proteolysis patterns as well as elution profile on gel filtration chromatography. There was, therefore, no evidence for a global change in the mutant enzyme. Thus, it appears that the activity of the enzyme is due to the functions of Cys-344 at or near the DNA-binding site.

Both wild-type and mutant enzymes that were active exhibited comparable kinetic parameters in $K_m$ and $k_{cat}$. The specificity constant ($k_{cat}/K_m$) which is a measure of enzyme efficiency was not drastically different for the wild-type and the mutant enzymes (Table II) except in the case of the C30S mutant enzyme. Although the C30S mutant enzyme was catalytically active, it is clear from Table II that the specificity constant for the enzyme was 5-fold less than the other mutant enzymes. This was mainly due to a 5-fold decrease in the turnover number of the enzyme. The kinetic data confirm the nonessential character of cysteines at positions 30, 213, 434, 553, and 577 in the catalytic mechanism. The striking loss of activity as measured by both in vivo and in vitro assays argues strongly for a specific role for cysteine 344. To confirm this conclusion further, double and triple mutants were generated, some of which had the cysteine at position 344 replaced with serine. Again, all double and triple mutants that had a serine at position 344 did not show wild-type activity, whereas all other combinations did not result in loss of methylation activity (Fig. 7). Collectively, these results strongly suggest that cysteine 344 plays a significant role in enzyme function. Substitution of a single amino acid residue can sometimes result in a decrease of enzyme activity, even if the residue is not involved in the active site. This could be due to changes in the higher order protein structure. Such critical residues play a basic role in protein folding and/or in supporting correct protein structure. Replacement of such residues could lead to decreased protein stability and also to a decreased level of expression due to higher accessibility of mutant proteins to proteases. Similar results obtained on the oligomeric nature of the wild-type and C344A mutant enzyme suggest that loss of methylation activity in the case of the latter was not due to changes in the higher order protein structure.

The structural similarity among the active sites of M.PvuII, M.TaqI, and M.HhaI reveals that catalytic amino acids essential for cytosine N-4 and adenine N-6 methylation coincide spatially with those for cytosine C-5 methylation suggesting a mechanism for amino methylation. Based on the chemical and structural similarity of the DNA-adenosyl and AdoMet-adenosyl moieties and the structural similarity of the AdoMet binding and catalytic regions of the MTase, Malone et al. (18) have proposed analogous MTase-adenosine interactions in the two regions. Chemical model studies suggest that methyl transfer reactions with amine nucleophiles and methyl sulfonium compounds require significant activation (45, 46). Malone et al. (18) have proposed a model that suggests that methylation of the exocyclic amino group results from a direct attack of the activated adenine N-6 on the AdoMet methyl group, in analogy with the previously proposed mechanism for DNA adenine methylation (47–49). It has been suggested that the N-6 amino nitrogen of the target adenine is the donor in a hydrogen bond to the side chain of aspartic acid in motif IV and possibly to one of the main chain oxygens of the adjacent two proline residues. This would negatively polarize N-6, activating it for direct transfer of the CH$_3$ from AdoMet. The relative positions of the activating hydrogen bond acceptor, target amino group, and AdoMet methyl group must be precisely maintained. Although it has been suggested that increasing the nucleophilic nature of the exocyclic amines of adenine (N-6) with cysteine may be a common strategy for MTases, cysteine residues might, however, still have a role as general base in catalysis of the methylation. It was earlier observed that few adenine MTases contain a cysteine flanked by asparagine akin to a PC doublet seen in m5C-MTases (27). Whereas it is firmly established that the PC motif is the catalytic center in m5C-MTases, no such role has been identified for the CN or NC motif seen in few m6MTases. In any case, such dipeptide sequences are not present in the case of EcoP15I DNA MTase.

The key finding of the present work is the identification of the single cysteine in M. EcoP15I involved in DNA binding and that Cys-344 is modified by NEM. Taken together, our experiments unambiguously show the consequences of replacing the cysteine residue at position 344 with alanine or serine. Whether this residue actually constitutes part of the active site or influences that site indirectly through conformational mechanisms will be the subject for future studies.

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