Cytosine Methyltransferase from *Escherichia coli* in Which Active Site Cysteine Is Replaced with Serine Is Partially Active†

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ABSTRACT: *Eco*RII methyltransferase (M.*Eco*RII) catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to C-5 position of second cytosine in the DNA sequence 5′-CCWGG (W = A or T). The reaction is initiated by a nucleophilic attack of the C-6 of target cytosine by a cysteine that is conserved among all cytosine methyltransferases. We have replaced this cysteine in M.*Eco*RII with serine or alanine and purified the proteins to homogeneity. The catalytic efficiency (k_{cat}/K_m) of the mutant enzyme with serine (C186S) for methyl transfer is about 10 000 times less than that of WT but is substantially higher than the efficiency of the C186A mutant. We show that the WT enzyme and C186S mutant are proficient in exchange of proton at C-5 and that this activity is reduced in the mutant to the same extent as the methyl transfer activity. The C186S mutant is insensitive to a cysteine-specific inhibitor, and it transfers methyl groups to the same position of cytosine as the WT enzyme. The ability of serine to act as a nucleophile in the enzyme reaction suggests that it—and probably the cysteine in the WT enzyme—is activated by a nearby base. Like the WT enzyme, C186S forms stable SDS-resistant complexes with DNA containing 5-azacytosine; but unlike the WT enzyme, the mutant reacts faster with 5-azacytosine than with normal cytosine. Apparently, greater reactivity of 5-azacytosine assists the C186S mutant in catalysis.

DNA (cytosine-5) methyltransferases (C5 MTases) are a class of enzymes that catalyze one-carbon transfer to position 5 of cytosine in DNA by an addition/elimination mechanism. This class of enzymes is related in its catalytic mechanism to other enzymes that carry out methyl group transfer to pyrimidines. These include thymidylate synthase (Santi & Danenberg, 1984), dUMP hydroxymethylase (Kunitani & Santi, 1980), dCMP hydroxymethylase (Yeh & Greenberg, 1967), and tRNA (uracil-5) methyltransferase (Santi & Hardy, 1987). C5 MTases catalyze methyl group transfer from *S*-adenosylmethionine (SAM) to cytosines within specific DNA sequences, producing 5-methylcytosine and *S*-adenosylhomocysteine (SAH).

Cytosine methylation is widespread in both prokaryotes and eukaryotes. Bacterial MTases are usually found as a part of restriction−modification systems and function in cell's defense against phage infection. Each bacterial C5 MTase recognizes a different DNA sequence and methylates a cytosine within that sequence. These enzymes share several conserved blocks of sequences and a common architecture (Lauster et al., 1989; Posfai et al., 1989). Most eukaryotic MTases methylate cytosines within CpG dinucleotides and contain a C-terminal segment that shares sequence with the bacterial enzymes (Bestor et al., 1988; Finnegan & Dennis, 1993; Yen et al., 1992). These enzymes play important roles in the regulation of gene expression and cellular differentiation. Moreover, sites of cytosine methylation are known as hot spots for cytosine to thymine mutations (Cooper & Youssoufian, 1988; Coulondre et al., 1978; Lieb, 1991; Wyszynski et al., 1994). Because of their biological importance, their sequence specificity, and the similarity between their reaction and the mechanism of other nucleotide modifying enzymes, C5 MTases have been the subject of many studies in the past decade. Most studies were done with bacterial MTases, and model enzymes have been studied to understand the mechanism of action of all C5 MTases.

Several lines of evidence suggest that the methylation reaction proceeds as outlined in Scheme 1 (Santi et al., 1983). There are two key species (**2a** and **3**) in this mechanism. The existence of species **2a** in the reaction has been demonstrated using an assay that detects enzyme-catalyzed exchange of proton at C-5 [Scheme 1 and Wu and Santi (1985, 1987)] . Existence of intermediate **3** in the reaction has been demonstrated with the use of mechanism-based inhibitors. DNA containing 5-azacytosine or 5-fluorocytosine at the target site irreversibly inactivates the MTases. These cytosine analogs inhibit the β-elimination step and hence trap the covalent intermediate **3** in the reaction (Santi et al., 1983). Using DNA containing these analogs, intermediate **3** formed by several bacterial enzymes (Chen et al., 1991; Friedman, 1985; Friedman & Ansari, 1992; Gabbara & Bhagwat, 1995; Hanck et al., 1993; Osterman et al., 1988; Santi et al., 1984; Wyszynski et al., 1993) and one eukaryotic enzyme (Christman et al., 1985; Smith et al., 1992) has been trapped.

The thiol in the only conserved cysteine among C5 MTases carries out the attack at C-6. This has been shown genetically and biochemically. Substitution of this amino acid with

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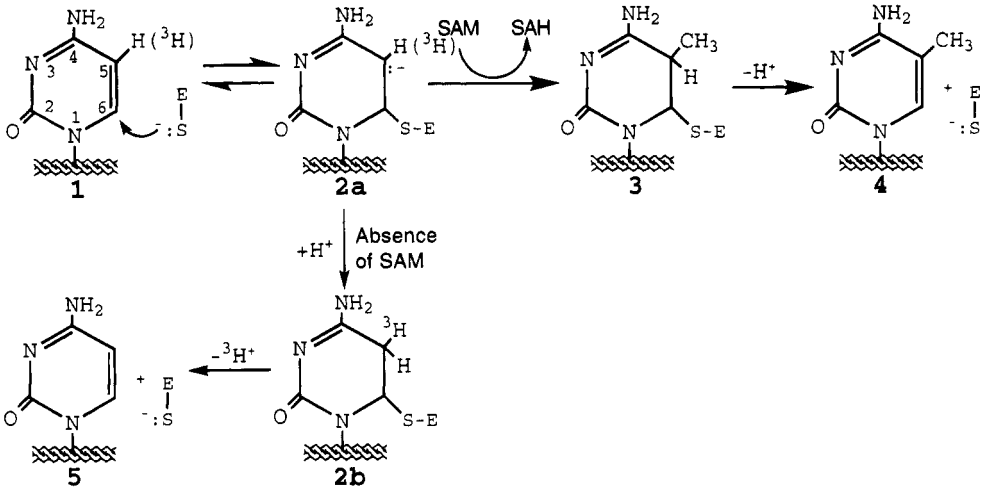
Scheme 1. Mechanisms of Methyl Transfer and Tritium Exchange Reactions[a]



[a] In experiments done to study 5-H exchange, carbon-5 was tritiated. For this reason, ^3H is shown in parentheses at that position.

others results in a substantial loss of MTase activity (Hanck et al., 1993; Mi & Roberts, 1993; Wilke et al., 1988; Wyszynski et al., 1992; Wyszynski et al., 1993) and an inability to form stable complexes with DNA containing 5-fluorocytosine (Chen et al., 1993; Hanck et al., 1993; Wyszynski et al., 1993). Covalent intermediate **3** formed by two wild-type bacterial MTases with DNA containing 5-fluorocytosine has been purified and analyzed by proteolysis (Chen et al., 1991; Friedman & Ansari, 1992). The DNA was found to be attached to a peptide that contains the conserved cysteine, and the cysteine was found to be alkylated (Chen et al., 1991; Friedman & Ansari, 1992).

We were interested in finding out if replacement of the catalytic cysteine in a C5 MTase by a potentially nucleophilic serine could result in a partially active enzyme. We have previously described (Wyszynski et al., 1992, 1993) such a mutant of *Eco*RII MTase (M.*Eco*RII). This enzyme methylates the second cytosine in the sequence 5′-CC(A or T)GG (May & Hattman, 1975; Schlagman et al., 1976). While one study of this mutant of M.*Eco*RII (Wyszynski et al., 1992) and of a similar mutant of another *E. coli* MTase, Dcm (Hanck et al., 1993), had suggested that these mutants may be partially active, other studies of the M.*Eco*RII mutant (Wyszynski et al., 1993) and of other MTases (Chen et al., 1993; Mi & Roberts, 1993) had indicated that this may not be the case. To clarify this, we purified the mutant of M.*Eco*RII containing cysteine to serine substitution to homogeneity and compared its properties with those of the wild-type (WT) enzyme and of a mutant with alanine substitution at the same position.

MATERIAL AND METHODS

Protein Purification. The host for the overproducing plasmids carrying the genes for the wild-type M.*Eco*RII and its mutants was the *Escherichia coli* B strain BL21 (DE3) (F^- *ompT hsdS*) with a phage λ lysogen (*imm21 int*) which contains T7 RNA polymerase gene under the *lac* UV5 promoter (Studier et al., 1990). The plasmid constructs pT71-Cys and pT71-Ser carrying the genes for the WT enzyme and the C186S mutant, respectively, have been described before (Wyszynski et al., 1992). The overproducer pT71-Ala carrying the gene for the C186A mutant is similar to the above constructs (M. W. Wyszynski and A. Bhagwat, unpublished results). The C186A mutant was coexpressed in the cell with the *Mva*I MTase, which is expressed from the multicopy number plasmid, pBC1018 provided by A. Janulaitis (Fermentas, Vilnius, Lithuania), to help obtain high yield of the C186A mutants (see Results).

The protein was purified from *E. coli* BL21(DE3) harboring the appropriate overproducer. A total of 2 L of culture was grown to an OD_{550} between 0.3 and 0.4 before induction with 120 μg/mL isopropyl β-D-thiogalactoside. Growth was continued for another 3−4 h, and cells were collected by centrifugation in a Sorvall GSA rotor at 4000*g* for 20 min. Three to four grams of cells was resuspended in 50 mL of "medium salt buffer", buffer A (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 10 mM 2-mercaptoethanol), with 0.1 M NaCl, and the first purification step with cellulose phosphate was carried out as described earlier (Wyszynski et al., 1993). The enzyme after this step appeared to be substantially pure (~70% homogeneous). Most impurity appeared was due to the copurification of the lysozyme that was added during the preparation of the cell-free extract. The pooled fractions (20−30 mL) containing MTase activity were dialyzed once against buffer A with 0.15 M NaCl and twice against "low salt buffer", buffer A with 0.05 M NaCl. The desalted aliquot was centrifuged in a SS-34 rotor (Sorvall) at 10000*g* for 15 min, and the supernatant was loaded onto DEAE Sephacel (Pharmacia-LKB, Piscataway, NJ) column (1.6 × 20 cm) pre-equilibrated with the low salt buffer. The column was washed with 40 mL of the low salt buffer at 0.5 mL/min, and the enzyme was eluted from the column with a linear gradient of 0.05−0.4 M NaCl in buffer A. The MTase and its mutants eluted as a single peak at about 0.2 M NaCl. Fractions (about 20 mL) within this peak were pooled and concentrated to 2 mL by filtering through a YM10 disc membrane in an Amicon ultrafiltration stirred cell (Amicon, W. R. Grace & Co., Danvers, MA). The concentrated aliquot was loaded onto an FPLC Superdex^{75} (Pharmacia-LKB), and the enzyme was eluted with buffer A containing 0.2 M NaCl at 0.5 mL/min. The MTase and its mutants eluted from this gel-filtration column as a single peak at 50−60 mL. Fractions within this peak were pooled and loaded onto a heparin-Sepharose (Pharmacia-LKB) column (0.8 × 10 cm) pre-equilibrated with buffer A containing 0.2 M NaCl. The enzyme was eluted from the

Table 1: Sequences of the Duplexes

Duplex	Base X	Sequence‡
I*	-	5'GGGG.....ATCCCAGGAAT.....TT CCCC.....TAGGGTCCTTA.....AAGATC
II	-	5'GTCTGCGACAGATTC CAGACGCTGTCTAAGGACCCTATTC me
III	Cytosine	5'GTCTGCGACAGATTCXTGGGATAAG 3'CAGACGCTGTCTAAGGACCCTATTC me
IV	[5-^{3}H] Cytosine	same as III
V	Cytosine with ^{32}P at 5' side	same as III
VI	5-azacytosine	same as III

* Complete sequence of duplex I is given in Gabbara (1992). ‡ The EcoRII site is underlined for emphasis. "me" represents the methylation at position 5 of cytosine.

column with buffer A containing 0.5 M NaCl. Fractions containing the MTase were concentrated to 0.15−0.2 mg/mL by filtering through a YM10 disc membrane and dialyzed overnight against medium salt buffer and for 5 h against 45% glycerol solution containing 10 mM potassium phosphate, pH 7.0, 50 mM NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol. After dialysis, bovine serum albumin was added to a final concentration of 200 μg/mL, and the purified enzyme was stored at −20 or −70 °C. The WT and the mutant proteins were >99% pure as judged by SDS−PAGE stained with Coomassie Brilliant Blue solution.

Formation of Oligonucleotide Duplexes Containing Single EcoRII Sites. All oligonucleotide DNA duplexes were synthesized by the Macromolecular Facility (Wayne State University School of Medicine) and are listed in Table 1. Duplex I was described earlier (Gabbara & Bhagwat, 1992). Synthesis of duplexes III and VI have been also been described earlier (Gabbara & Bhagwat, 1995). The latter duplexes were synthesized by extending the 15-mer primer of duplex II using dATP, dTTP, and dGTP, either dCTP for duplex III or (5-aza) dCTP (provided by S. Friedman, State University of New York, Brooklyn) for duplex VI, and Sequenase T7 DNA polymease version 1.0 (United States Biochemical) in the recommended buffer. Duplexes IV and V were synthesized similarly except that 10 μM [5-^{3}H]dCTP [specific activity: 17.3 Ci/mmol (Amersham Corp.)] for duplex IV, or 5.8 μM dCTP + 0.8 μM [α-^{32}P]dCTP (specific activity: 3000 Ci/mmol (Du Pont-New England Nuclear)] for duplex V was used. The creation of *Eco*RII site was confirmed by *Bst*NI restriction digest.

Methyltransferase Assays. The specific activities of the WT M.*Eco*RII and its mutants were quantitated by measuring the transfer of methyl groups from *S*-[*methyl*-^{3}H]adenosyl-L-methionine ([*methyl*-^{3}H]SAM) (72.5 Ci/mmol) (NEN/Dupont, Boston, MA) to chromosomal DNA isolated from *E. coli* RP4182 lacking cytosine methylation. Reactions were carried out in an 80-μL volume of MTase buffer (100 mM Tris-HCl pH 7.8, 20 mM EDTA, pH 8.0, 0.4 mM dithiothreitol) at 37 °C and contained DNA (2.0 μg), RNaseA (4 μg), [*methyl*-^{3}H]SAM (30 pmol), 8.9 ng of the WT enzyme, 3.7 μg of the C186S mutant, or 3.9 μg of the C186A mutant. Reactions were stopped at different time intervals by adding SDS to 0.5% and heating at 70 °C for 5 min. Unincorporated [*methyl*-^{3}H]SAM was removed using spun columns (Bhagwat, 1993) of Sepharose CL-6B (Pharmacia-LKB). Quantitation of radioactivity was measured by scintillation counting.

Steady-state kinetics of methyl transfer was performed using duplex III as substrate. For the WT enzyme the reaction contained different concentrations of duplex III (1.6−90 nM), [*methyl*-^{3}H]SAM (72.5 Ci/mmol) (0.8 μM) and 2.1 nM of enzyme. For the C186S mutant the reaction contained 14.8−220 nM duplex III, [*methyl*-^{3}H]SAM (72.5 Ci/mmol) (1.5 μM) and 85 nM mutant protein. Reactions were performed for 1 and 2 min at 37 °C in MTase buffer. Reactions were terminated by adding SDS to 0.5% and treated with proteinase K (f.c. = 0.1 mg/mL) at 50 °C for 1 h. Unincorporated [*methyl*-^{3}H]SAM was removed using G-50 (Pharmacia-LKB, Piscataway, NJ) spun columns. Radioactivity was quantitated by scintillation counting. Reaction rates determined at different DNA concentrations were analyzed using the Stat View 512+ package for the Macintosh to calculate the kinetic constants for this reaction.

The time-course assay which compared MTase activity of the C186S mutant to either azaC DNA or to normal DNA was performed by mixing duplex VI (0.4 pmol) or duplex III (0.3 pmol) with 13 pmol of [*methyl*-^{3}H]SAM (83.9 Ci/mmol) and 8.5 pmol of the mutant enzyme in a final volume of 20 μL. Reactions were terminated at different time interval by adding SDS to final concentration of 0.5% and treated with proteinase K (f.c. = 0.1 mg/mL) at 50 °C for 1 h. Removal of unincorporated [*methyl*-^{3}H]SAM and quantitation of radioactivity was done as described above.

Methylation of duplex I by the WT enzyme and the C186S mutant was done as follows. The 42-mer duplex was radioactively end-labeled with [γ-^{32}P]ATP as described previously (Gabbara & Bhagwat, 1992). A 2-fold excess of cold 46-mer was added, the mixture was heated at 90 °C for 3 min, and the two strands were allowed to anneal by cooling the mixture until it reached 25 °C. The solution was extracted once with phenol/chloroform and once with chloroform and was spun-dialyzed through Sephadex G-50 column to remove unincorporated [γ-^{32}P]ATP. Eighteen picomoles of end-labeled duplex I was incubated with 1.4 μM SAM, either 25.5 pmol of WT M.*Eco*RII or 51 pmol the C186S mutant in MTase buffer in a 120 μL reaction volume at 37 °C for 18 h. After the first 6 h, the reaction mixture containing the mutant enzyme was extracted with phenol/chloroform and was spun-dialyzed through Sephadex G-50. Then fresh enzyme and SAM were added. At the end of the 18-h incubation the phenol/chloroform extraction and spin-dialysis were repeated for both the WT and the mutant enzyme. Control reactions were performed as described above except that no MTase was added. Methylation of the DNA duplex was tested by challenging it with *Eco*RII and *Mva*I endonucleases. Restriction digests by these enzymes were performed by incubating 1 pmol of the DNA duplex with 4.5 units of *Eco*RII (this lab) or 12 units of *Mva*I (Amersham) in 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM 2-mecaptoethanol, and 50 mM NaCl (for *Eco*RII digest) or 150 mM NaCl (for *Mva*I digest), in a 30-μL reaction volume for 2 h at 37 °C. For the *Eco*RII digest, 0.18 pmol of plasmid pUC119 was included to activate the enzyme for cutting (Gabbara & Bhagwat, 1992). The products of the restriction digests were separated by elec-

Table 2: Kinetic Constants and Specific Activities of Purified Proteins

	methyl transfer				tritium exchange		
phenotype	specific activity[a]	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (M^{-1} S^{-1})	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (M^{-1} S^{-1})
wild type	5890	0.38	15.1	4.19×10^5	10	216	7.72×10^5
C186S mutant	0.065	1.7×10^{-5}	6.8	36.8	1.6×10^{-3}	459	58.1
C186A mutant	0.0015	ND	ND	ND	ND	ND	ND

[a] Specific activity is defined as the number of picomoles of methyl groups transferred to chromosomal DNA per min per mg of protein. [b] ND, values not determined.

trophoresis in a 10% polyacrylamide gel prepared in TBE (90 mM Tris, 90 mM boric acid, and 2 mM EDTA; final pH 8.1) electrophoresis buffer.

Tritium Exchange Assay. Various amounts of duplex IV and enzyme were incubated in MTase buffer in a total volume of 80 μL. Reactions were carried out at 37 °C for different time intervals and were quenched by adding 10 μL of 10 mg/mL chromosomal DNA isolated from hering sperm (Boehringer Mannheim) and 10 μL of 100% trichloroacetic acid. The reaction mixture was chilled on ice for at least 30 min to allow precipitation of protein and DNA and was spun in a microfuge for 15 min. The pellet which contained the precipitated protein and DNA was discarded. Additional 10 μL of the 10 mg/mL chromosomal DNA was added to the supernatant, and the precipitation and centrifugation steps were repeated. The radioactivity in the supernatant, which would contain tritium released from the duplex, was quantitated by scintillation counting. Steady-state kinetics for the WT enzyme was conducted at substrate concentrations between 6.25 and 450 nM, using 2 nM enzyme. For the C186S mutant steady-state kinetics was conducted at substrate concentrations between 112 and 900 nM, using 42.5 nM enzyme. Reactions were performed for 1 and 2 min and for 30 min for the WT enzyme and the C186S mutant, respectively. Reaction rates determined at different substrate concentrations were analyzed using the Stat View 512+ package for the Macintosh to calculate the kinetic constants for this reaction.

Reaction of Methyltransferase with 5,5′-Dithiobis(2-nitrobenzoic Acid). The concentration of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in stock solutions was determined by titration with free cysteine using the molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm. DTNB (0.2 mM) was reacted with enzyme (50 nM) in a total volume of 1 mL of MTase buffer at 25 °C. Aliquotes (100 μL) were withdrawn at different time intervals, and the reaction with DTNB was stopped by adding 3.9 μL of a solution containing 0.51 M 2-mercaptoethanol and 2.8 μg/μL bovine serum albumin. A tritium exchange assay was used to measure the enzyme activity in the resulting solution. Tritium exchange was initiated by adding 1 or 2 μL of duplex IV (~0.9 pmol/μL) to the solution containing the WT enzyme or the C186S mutant, respectively. The reaction was allowed to proceed for 1 min (for the WT enzyme) or 2.5 h (for the C186S mutant). Reaction conditions and quantitation of released ^3H were done as described above.

Covalent Complex Formation with 5-Azacytosine-Containing DNA. Oligonucleotide DNA duplex VI was radioactively end-labeled with [γ-^{32}P]ATP as previously described (Gabbara & Bhagwat, 1992). For each reaction, 0.2 pmol of the labeled duplex, 25 pmol of mutant enzyme, and 3 nmol of SAM were mixed in MTase buffer in a final volume of 30 μL. The reaction mixtures were incubated at 37 °C for 2 h. SDS was then added to a final concentration of 0.8%. One half of the resulting mixture was electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS in both the gel and the electrophoresis buffer. The other half was heated at 70 °C for 5 min prior to electrophoresis. Electrophoresis was carried out at 25 °C at 150 V for 4 h. The gels were exposed to Fuji X-ray film RX for 1.5 h.

RESULTS

Purification of M.EcoRII and Its Mutants. The WT M.*Eco*RII and its mutants C186S and C186A were purified to apparent homogeneity using four chromatography steps as described in Materials and Methods. While the hosts from which the WT enzyme and C186S mutant were purified were the same, a different host had to be used for the purification of C186A. We have previously shown that some of the mutants of M.*Eco*RII in which cysteine-186 has been substituted with other amino acids are toxic to *E. coli* (Wyszynski et al., 1992). In particular, the C186G mutant causes inhibition of cell growth (Wyszynski et al., 1992). A similar inhibition was also seen with the C186A mutant (M. Wyszynski and A. Bhagwat, unpublished results). The growth arrest is thought to be due to abnormally stable DNA binding by the mutant MTase, which probably interferes with essential cellular processes (Mi & Roberts, 1993; Wyszynski et al., 1992).

To overcome this growth inhibition, the gene encoding the C186A allele was expressed in cells in which *Mva*I methyltransferase (M.*Mva*I) was also present. M.*Mva*I methylates the N-4 position of the same cytosine that M.*Eco*RII methylates, and methylation at N-4 is known to inhibit methylation at C-5 by M.*Eco*RII (Butkus et al., 1985). We speculated that prior methylation of DNA by M.*Mva*I may prevent DNA-binding by M.*Eco*RII and hence alleviate growth inhibition. This was found to be the case (not shown). Mi and Roberts (1993) used a similar strategy to overcome toxicity due to the C81G mutant of M.*Hha*I. It should be noted that the methyltransferase activity due to M.*Mva*I was largely removed during the first chromatography step and was eliminated in the subsequent purification steps (not shown).

C186S Mutant Is Weakly Proficient in Methyl Transfer. In an earlier study, we showed that C186S mutant binds tightly and specifically to substrate DNA and that a partially purified preparation of the mutant was at least 10 000-fold lower in MTase activity than the WT enzyme (Wyszynski et al., 1993). The specific activities of the purified C186S and C186A mutants were 9.1×10^4 and 3.9×10^6 times lower than that of the WT enzyme (Table 2). Although the MTase activity of both mutants were low, the activity of

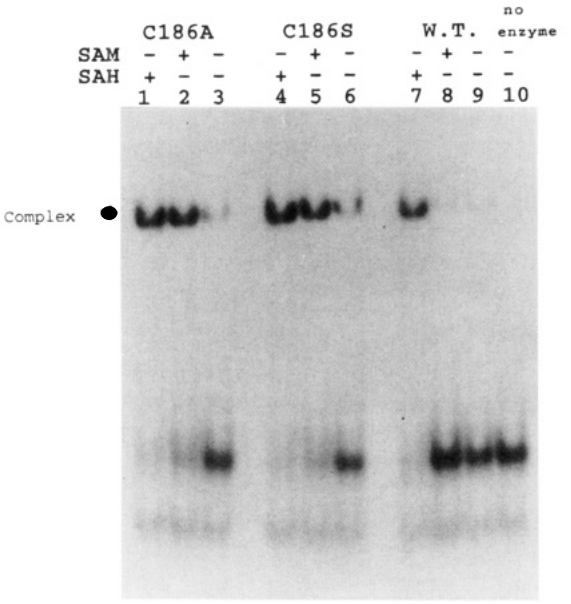


FIGURE 1: Binding of C186A mutant to substrate DNA. WT enzyme, C186S mutant, or C186A mutants were incubated with labeled duplex III and electrophoresed through a polyacrylamide gel under nondenaturing conditions. The reactions contained 1.8 pmol of duplex and 8.5 pmol of WT enzyme, or 17 pmol of mutant protein and 3 ng of poly(d-IC) DNA. Incubations were carried out at 37 °C for 30 min without any cofactor ("−" sign above the lanes) or with 10 μM concentration of the indicated cofactor ("+" sign).

C186S was substantially higher than that of C186A. In one experiment, unmethylated chromosomal DNA was incubated with similar amounts of C186S or C186A, and the transfer of tritiated methyl groups was measured. In a 1 h reaction, the amount of ^{3}H in DNA increased to 30-fold above background with C186S but was only slightly above background with C186A (not shown). It should be pointed out that the inability of C186A to transfer methyl groups is not due to a defect in subtrate-binding. C186A binds tightly to duplex III (Table 1), and this binding is stimulated by the presence of SAM or SAH (Figure 1). The binding of C186A to its cognate sequence is also predicted by the genetic evidence regarding its toxicity (see above).

Steady-state kinetic constants for C186S (Table 2) suggest that the mutant is impaired in catalysis, but not in affinity to the substrate. While the turnover number for the mutant is approximately 22 000 times lower than WT, in so far as K_m may approximate K_S, the apparent affinities of the two proteins to substrate DNA are similar (Table 2). Recently, we confirmed the latter result by determining the K_d of the WT enzyme and of the C186S mutant for their DNA substrate in the absence of cofactor and in the presence of SAH. The K_d values for the two proteins were found to be similar (not shown). These results show that the substitution of the reactive thiol group with a hydroxyl group has only a small effect on substrate binding.

C186S Mutant Does Not Methylate N-4 of Cytosine. Because of the low MTase activity of C186S mutant, it seemed possible that the observed methyl transfer may be to a different target than what is used by the WT enzyme. In particular, it seemed possible that serine-186 may not directly participate in catalysis, and the observed methyl transfer to DNA may be due to a direct attack at the N-4 position of cytosine by the methyl group in SAM. Enzymes like M.*Mva*I catalyze methyl transfer to N-4 of cytosine, and

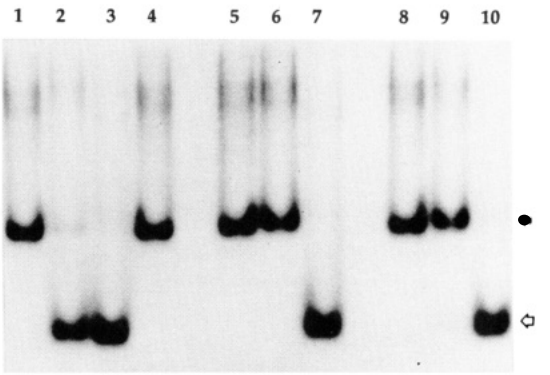


FIGURE 2: Specificity of methylation of the C186S mutant. One of the strands of duplex I was end-labeled with ^{32}P and was incubated in MTase buffer with SAM and either WT M.*Eco*RII (lanes 5−7) or C186S mutant (lanes 8−10) or without MTase (lanes 1−3). For the sample in lane 4, no incubation was carried out. The DNA was then loaded onto the gel (lanes 1, 4, 5, and 8), or it was subjected to digestion reactions with *Eco*RII (lanes 2, 6, and 9) or *Mva*I (lanes 3, 7, and 10) endonucleases. Substrate DNA and the labeled product are indicated by solid and open arrows, respectively.

they do not appear to have a conserved cysteine within their catalytic site (Lauster, 1989; Wilson, 1992). To eliminate this possibility, we methylated a DNA substrate (duplex I, Table 1) with the mutant and challenged the DNA with *Eco*RII endonuclease (R.*Eco*RII) or *Mva*I endonuclease (R.*Mva*I). Although both endonucleases cleave within the *Eco*RII recognition sequence, they are sensitive to different cytosine methylations. While R.*Eco*RII is inhibited by either methylation (Butkus et al., 1985; May & Hattman, 1975; Schlagman et al., 1976), R.*Mva*I is inhibited by N-4 methylation, but not by C-5 methylation of cytosine (Butkus et al., 1985). We found that DNA methylated by WT enzyme or by C186S mutant was resistant to *Eco*RII (Figure 2, lanes 6 and 9) but sensitive to *Mva*I (Figure 2, lanes 7 and 10). About 80% of the DNA methylated with C186S was susceptible to R.*Mva*I, and about 86% of the same DNA was resistant to R.*Eco*RII. This is consistent with the transfer of methyl groups by C186S to C-5 of cytosine, but not to N-4.

C186S is Defective in Attack of C-6 of Cytosine. Wu and Santi (1985, 1987) showed that M.*Hha*I can cause exchange of proton at C-5 of target cytosine and that this was the consequence of formation of a Michael adduct by the enzyme (structure **2a**, Scheme 1). We used a substrate with ^{3}H at C-5 position of the target cytosine (duplex IV, Table 1) to study 5-H exchange and measured the steady-state constants of WT and C186S for this reaction.

In the absence of cofactor, M.*Eco*RII caused a rapid release of ^{3}H in a time-dependent fashion (Figure 3A). This reaction is ~25 times faster than the methyl transfer reaction (Table 2). Thus, tritium release is a more sensitive assay for catalytic studies of M.*Eco*RII than methyl transfer. Consistent with earlier results with M.*Hha*I (Wu & Santi, 1985, 1987), ^{3}H release by the enzyme was inhibited by SAH (Figure 3B). For M.*Eco*RII, 50% inhibition was seen at a SAH concentration of about 100 nM. When the tritiated substrate was incubated with an execess of mutant with serine substitution, significant amounts of tritium were released over a 2-h period (Figure 3A). In comparison, the ability of C186A mutant to release ^{3}H was at least 200-fold less than that of C186S (Figure 3A). To eliminate the possibility that radioactivity released from duplex IV by C186S was not the

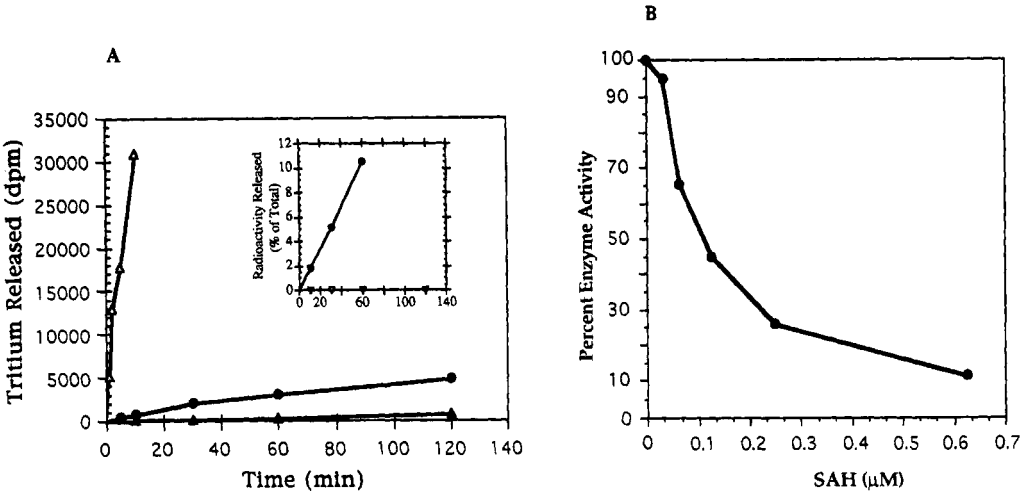


FIGURE 3: (A) Tritium release activities of WT M.*Eco*RII and its mutants. WT M.*Eco*RII (0.17 pmol), C186S mutant (3.5 pmol), and C186A mutant (71 pmol) were incubated with 2.5, 9, and 9 pmol of DNA duple IV, respectively, in MTase buffer in a final volume of 80 μL. Reactions were performed as described in Material and Methods. DNA and protein were precipitated and removed by centrifugation, and radioactivity in the soluble fraction was quantitated. Background radioactivity was 0.2% of total (64 dpm/pmol) for the tritiated substrate and 1% for the ^{32}P-labeled DNA and was subtracted from all values. Open triangles, WT enzyme; closed circles, C186S mutant; closed triangles, C186A mutant. (Inset) The assay was repeated with 3.7 μg of the C186S mutant and 4.5 pmol of duplex IV (closed circles) or 7.3 pmol of duplex V (inverted triangles). (B) Inhibition of ^{3}H release activity by SAH. Duplex IV (62 nM) was incubated with WT M.*Eco*RII (2.1 nM) in the presence of different amounts of SAH. Following 2 min incubations at 37 °C, the reactions were stopped and the release of ^{3}H was quantitated. The activity of the enzyme without SAH was 7.2 nM of ^{3}H/min and was defined as 100%.

result of degradation of the substrate by contaminating nuclease activity, the release of radioactivity by C186S from duplex V was studied. This substrate is identical to duplex IV except that it contains ^{32}P label in the phosphate preceding the target cytosine (Table 1). Under similar conditions, C186S released about 11% of the total radiaoctivity from substrate IV but released virtually no radiaoctivity from duplex V (Figure 3A, inset). ^{3}H release by the C186S mutant was also inhibited by SAH (not shown).

The k_{cat} of the C186S mutant in the ^{3}H release reaction is about 6000-fold less than the value determined for the WT enzyme, but the overall efficiency (k_{cat}/K_m^{DNA}) of this reaction is similar to that for methyl transfer (Table 2). Again the apparent affinity (K_m^{DNA}) of the mutant enzyme to DNA is not significantly different from that of the WT enzyme. We conclude that serine can substitute the active site cysteine for nucleophilic attack, although at a reduced efficiency. These results also show that the principal role of cysteine-186 in catalysis is as a nucleophile that attacks C-6 and it is unlikely to have roles in the subsequent steps in catalysis.

Catalytic Activity of C186S Is Insensitive to DTNB. Because the activity of C186S was low, it seemed possible that it could be the result of contamination by the WT enzyme during purification or due to occasional misreading of serine codon by a cysteinyl tRNA. To eliminate this possibility, we studied the effect of DTNB, a reagent known to react specifically with sulfhydryl groups, on the activity of the WT and the C186S proteins. Each protein was reacted with DTNB for different lengths of time, and the reactions were stopped by adding 2-mercaptoethanol. The residual activity in the proteins was determined using the ^{3}H-release assay. If cysteine-186 is the only cysteine in M.*Eco*RII that is critical for catalysis, then C186S mutant should be resistant to DTNB. This is what was observed. While the WT enzyme was quickly inactivated by DTNB, mutant with the serine substitution was not affected by it (Figure 4). The rate of inactivation of C186S mutant by DTNB was similar to its rate of inactivation in the absence of DTNB (Figure 4). These results show that the catalytical activity of C186S must be intrinsic to the protein and is unlikely to be due to contamination with WT enzyme.

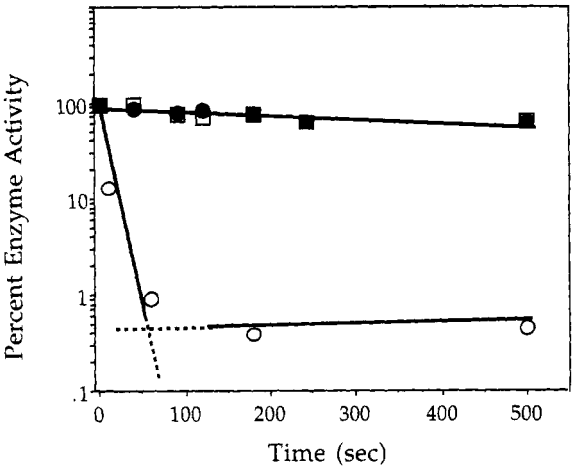


FIGURE 4: Effect of 5,5′-dithiobis(2-nitrobenzoic acid) on the activity of WT enzyme and the C186S mutant. WT enzyme or the mutant were reacted with DTNB, and the reactions were stopped with 2-mercaptoethanol at the indicated time points. The rate of ^{3}H release by the treated enzymes has been plotted as a function of time of incubation with DTNB. Open circles, WT enzyme with DTNB; closed circles, C186S mutant with DTNB; squares, C186S without DTNB.

C186S Is More Reactive toward 5-Azacytosine-Containing DNA than Normal DNA. We confirmed the ability of the C186S mutant to form a Michael adduct with the substrate by a second method. DNA containing the mechanism-based inhibitor 5-azacytosine (azaC DNA) was used to trap the Michael adduct (duplex VI, Table 1). This duplex is similar to duplex III except that the target base is 5-azacytosine instead of normal cytosine. We recently studied the interaction of the WT enzyme with this duplex in the presence of SAM (Gabbara & Bhagwat, 1995). We found that the enzyme forms a stable complex with this inhibitor and transfers a methyl group to it (Gabbara & Bhagwat, 1995).

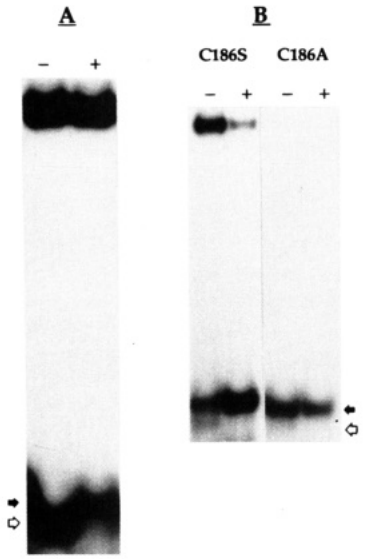


FIGURE 5: Binding of WT M.*Eco*RII and its mutants to azaC DNA. Duplex VI (solid arrow) was synthesized by the polymerization of duplex II (open arrow) in the presence of 5-azadCTP. ^{32}P-labeled duplex VI was incubated with WT or mutant protein in the presence of excess SAM. Reactions were stopped by adding SDS to the mixture. One-half of each reaction was loaded on the gel without heating (lanes with "−" signs above them). The other half was heated prior to loading (lanes with "+" signs). (Panel A) WT enzyme. (Panel B) C186S and C186A mutants. Duplex II is not seen in samples that are heated because it dissociates. For simplicity, bands corresponding to single-stranded oligomers have been removed from the photograph. Panel A is based on data that have been presented before (Gabbara & Bhagwat, 1995).

Similar experiments were conducted to compare the reactivities of WT and the C186S and C186A proteins toward duplex VI. The duplex was end-labeled with ^{32}P and incubated with molar excess of different proteins in the presence of SAM. The reactions were terminated with the addition of SDS and one half of the mixture was electrophoresed on a polyacrylamide gel containing SDS. The second half was subjected to heating prior to loading onto the same gel. The WT enzyme forms an SDS- and heat-resistant complex with duplex VI (Gabbara & Bhagwat, 1995); Figure 5, panel A). While no complexes were observed with the C186A mutant (Figure 5, panel B), the C186S mutant formed a complex that was resistent to SDS (Figure 5, panel B). It should be noted that the complexes formed by the WT enzyme or by the C186S mutant with the normal DNA substrate are not resistant to SDS (not shown). These results strongly argue that C186S mutant, but not C186A mutant, is capable of forming a covalent complex with azaC DNA. The only difference between the complexes formed by WT enzyme and C186S mutant was that the complexes formed by the latter protein were more susceptible to dissociation when subjected to heating (Figure 5, panel B).

The ability of the serine mutant to form covalent complexes with azaC DNA also eliminates the possibility that its activity is due to contaminating WT enzyme. In the gel-shift assay (Figure 5), about 25 pmol of the serine mutant was able to shift nearly all of duplex VI (0.2 pmol). If this were due to contamination of the mutant with WT enzyme, at least 0.8% of the mutant preparation must be the WT enzyme. Clearly, this cannot be true because of the low methyl-transfer and 5-^{3}H release abilities of the serine mutant (Table 2).

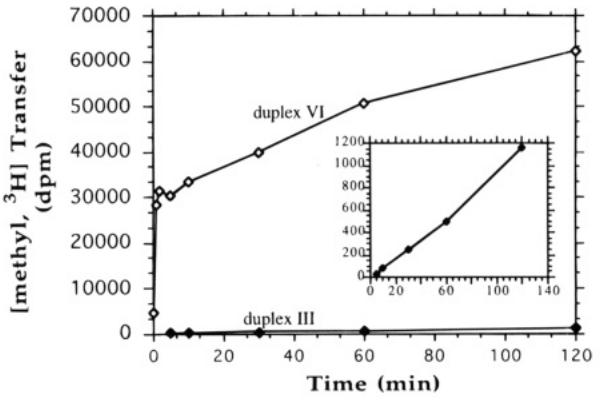


FIGURE 6: Methyl transfer to azaC DNA catalyzed by the C186S mutant. The rates of methylation of DNA duplexes III and VI are presented. C186S mutant was incubated with similar amounts of duplex III or VI and tritiated methyl groups transferred to the DNA were quantitated. (Inset) The amount of radioactivity transfered by C186S to duplex III is shown on an expanded scale.

Like WT enzyme (Gabbara & Bhagwat, 1995), C186S mutant is also able to transfer a methyl group to azaC DNA prior to its inactivation. Unlabeled duplex VI was reacted with C186S in the presence of [*methyl*-^{3}H]SAM, the resulting complexes were dissociated by heating the sample, and the mixture was electrophoresed in a polyacrylamide gel. Gel slices corresponding to the position of the free DNA were removed from the gel and quantitated for the presence of ^{3}H. It was found that ~50% of total DNA used in the the reaction was methylated by C186S during a 1-h reaction (not shown). This was a much higher rate of methyl transfer than would be predicted from studies with the normal substrate (Table 2). To confirm this finding, we compared the kinetics of methyl transfer by C186S to normal DNA (duplex III) and azaC DNA (duplex VI). Interestingly, duplex VI was methylated at an initial rate that was ~2400 times the rate of methylation of duplex III (Figure 6). Clearly, 5-azacytosine containing substrate was much more reactive toward C186S than normal DNA. We have not seen similar rate enhancement with azaC DNA when the WT enzyme was used for methyl transfer (not shown). We suggest that 5-azacytosine assists the C186S mutant in catalysis (see below).

DISCUSSION

We have shown that when the active site cysteine in M.*Eco*RII is replaced by serine, the activity of the enzyme is substantially reduced, but not eliminated. M.*Eco*RII is the first C5 MTase in which a cysteine to serine substitution has been shown to have detectable enzymatic activity. In most previous studies of C5 MTases in which the catalytic cysteine was substituted with serine, the lack of purity of the enzyme (Wilke et al., 1988; Wyszynski et al., 1993) or the nature of the assay (Chen et al., 1993) prevented a critical evaluation of the enzymatic activity of the mutant. Only the mutant of Dcm with serine substitution was found to have MTase activity that was about 1000 times lower than the WT enzyme (Hanck et al., 1993). The significance of this small activity could not, however, be assessed because mutants with nonnucleophilic substitution were not studied, and the WT Dcm was found to have the unusual and unexplained ability to transfer methyl groups to itself (Hanck et al., 1993).

The ^{3}H-release experiments provide a direct measure to compare the ability of residues cysteine-186 and serine-186 in the two proteins to attack C-6 of target cytosine. k_{cat} values show that serine is about 6000 times weaker than cysteine in its reactivity (Table 2). However, the fact that serine can partially substitute for cysteine suggests that there should be a nearby basic residue which activates the hydroxyl group of serine. Such a residue could convert the hydroxyl into an activated alkoxide by abstracting its proton (pK_a ~16−18). Presumably, in the WT enzyme, this residue would convert the neutral thiol of cysteine into its conjugate base, the thiolate. Such a residue could not be identified in the crystal structure of M.*Hha*I with DNA (Klimasauskas et al., 1994). It is possible that it is not positioned in direct contact with the nucleophile, and a water molecule makes a bridge to the base catalyst. Alternately, methyl transfer by the enzyme causes a conformation change that may have moved this residue away from the cysteine. Interestingly, in the crystal structure of M.*Hha*I without DNA (Cheng et al., 1993) His-127 lies in close proximity of the catalytic cysteine and it may be responsible for the activation of the cysteine. Although this histidine is not conserved among all C5 MTases, it is present in several of these enzymes, including M.*Eco*RII.

The proposal for the catalytic mechanism of C5 MTases was fashioned after the mechanism of the better studied enzymes, thymidylate synthases (Santi et al., 1983). Interestingly, there is precedent in thymidylate synthases (TS) for the retention of enzymatic activity when active site cysteine is replaced by serine. Substitutions of the active site cysteine have been carried out for several TS including the enzymes from *E. coli*, bacteriophage T4, and *Lactobacillus casei* (Climie et al., 1990; Dev et al., 1988; LaPat-Polasko et al., 1990; Michaels et al., 1990). Mutant enzymes from *E. coli* and bacteriophage T4 were found to have retained 0.02% and 0.07% activity of the wild-type enzyme, respectively (Dev et al., 1988; LaPat-Polasko et al., 1990). Indeed, the activity of *E. coli* TS with serine was sufficient to partly complement *thyA* strains (Dev et al., 1988; Michaels et al., 1990). It is also interesting to note that T4 TS with serine formed SDS-resistant complexes with 5-fluoro-dUMP, although much less efficiently than the WT enzyme (LaPat-Polasko et al., 1990). A cysteine-to-Ala mutant was constructed only for the *E. coli* TS. Like the C186A M.*Eco*RII, TS with alanine was inactive.

Cysteine and serine proteases are among the best studied enzymes. Many of these are coded by animal viruses, and investigators have pointed out the structural similarity and the evolutionary relationship between virus coded proteases with cysteine at their catalytic site and cellular serine proteases such as chymotrypsin (Bazan & Flatterick, 1988; Gorbalenya et al., 1989). In all these enzymes, a nearby histidine is thought to activate the catalytic cysteine or serine. Several mutational studies have been carried out on the viral proteases, and in several cases mutants containing replacement of active site cysteine with serine are found to have retained considerable proteolytic activity. In two of these enzymes, the histidine that activates the cysteine has also been identified [see Dougherty and Semler (1993) for a review]. As pointed out above, our results also suggest that the catalytic cysteine in M.*Eco*RII may be activated by a nearby histidine. If true, the catalytic mechanism of C5 MTases would share features not only with thymidylate synthases but also with cysteine and serine proteases.

Wu and Santi (1985, 1987) pointed out that the ability of M.*Hha*I to cause 5-H exchange is direct evidence for the generation of a Michael adduct (species 2a, Scheme 1) by the enzyme. M.*Eco*RII is only the second C5 MTase for which 5-H exchange has been demonstrated. Both enzymes carry out 5-H exchange at a faster rate than methyl transfer. The k_{cat} value for ^{3}H release reaction for M.*Hha*I is about 7 times faster than the methylation reaction. These results show that the formation of species 2a cannot be the rate-limiting step in methyl transfer.

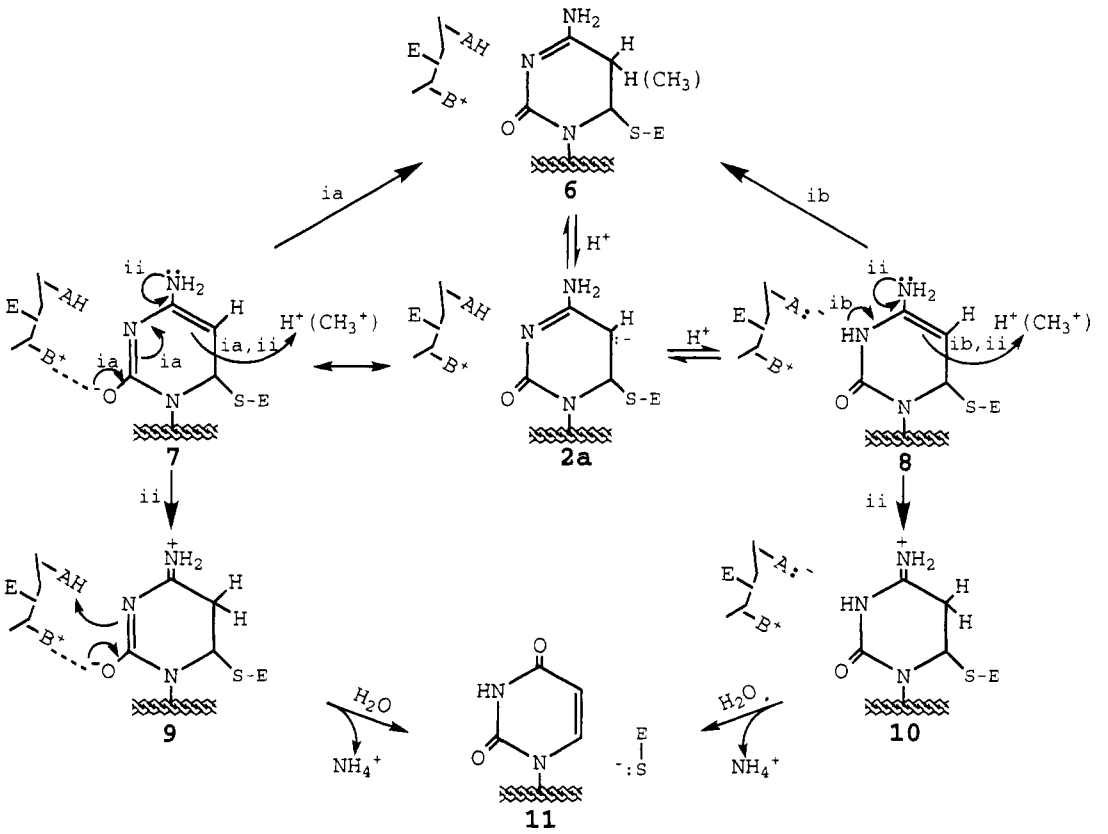
The precise pathway taken by the substrate to go from species 2a to intermediate **3** (Scheme 1) is unknown. Understanding the nature of the intermediates that occur after **2a** is important because the methylation reaction may share intermediates with other reactions of the enzyme such as 5-H exchange and deamination. The crystal structure of M.*Hha*I with DNA (Klimasauskas et al., 1994) provides several clues regarding these intermediates. It shows that the target cytosine flips completely out of the double helix and that the enzyme makes several hydrogen bond contacts with the target cytosine. These include hydrogen bonds between Glu-119 and Arg-165, and positions N-3 and O-2 of cytosine, respectively (Klimasauskas et al., 1994). Both of these residues are absolutely conserved in all C5 MTases (Lauster et al., 1989; Posfai et al., 1989). Verdine and his colleagues (Chen et al., 1993; Erlanson et al., 1993) pointed out that if N3 was in hydrogen-bond contact with the enzyme, the enzyme was likely to donate the proton to the substrate stabilizing the carbanion **2a**. They have further pointed out that the resulting delocalization of electrons in the heterocycle should create a reactive enamine functionality that could attack the methyl group in SAM.

We propose two possible pathways by which the delocalization could occur (Scheme 2). Electron delocalization could shift the negative charge from C-5 to O-2 creating the resonance structure **7**. The conserved arginine should stabilize this intermediate by forming a salt-bridge with O-2. Alternately, the conserved glutamate could donate a proton to N-3 forming the intermediate **8** (Scheme 2). The latter structure was previously suggested by Verdine and his colleagues (Chen et al., 1993; Erlanson et al., 1993). A subsequent protonation of the electron-rich intermediates **7** or **8** generates **6** (pathways ia and ib, Scheme 2) which could be cycled back to **2a**. Although it is possible that **6** can be formed directly from **2a**, we favor the hypothesis that it is formed via pathway i. It remains to be determined whether one or both **7** and **8** are formed during the reaction, and which one predominates.

As noted above, SAH inhibits 5-^{3}H release. This inhibition is likely to occur after the enzyme links to DNA; ie., the formation of **6** from **2a**, **7**, or **8** is blocked in the presence of SAH. We favor this explanation for the inhibition of the ^{3}H release reaction by SAH—rather than an inhibition of nucleophilic attack of C-6—because in recent studies we showed that WT M.*Eco*RII can covalently link to DNA containing 5-fluorocytosine (Wyszynski et al., 1993) or 5-azacytosine (Gabbara & Bhagwat, 1995) in the presence of SAH. Thus SAH may be a useful tool to study intermediates in the methyl transfer reaction.

Recently, we and others have shown that C5 MTases catalyze cytosine to uracil deaminations at sites of methylation (Shen et al., 1992; Wyszynski et al., 1994). Since SAH

Scheme 2. Intermediates in 5-H Exchange, Methyl Transfer, and Deamination Reactions



inhibits this reaction as well (Shen et al., 1992; Wyszynski et al., 1994), and since the C186A mutant of M.*Eco*RII was unable to cause cytosine deamination (Wyszynski et al., 1994), the mechanisms of deamination and 5-H exchange may be linked. We propose that **7** or **8** is the common branch point for both events. Both the intermediates contain an enamine functionality which tautomerizes to iminium group with simultaneous protonation forming **9** or **10** (pathway ii, Scheme 2). In principle **7** can also isomerize to **8**. Hydrolysis of iminium group in **9** or **10** would form uracil (structure **11**, Scheme 2). It should be noted that **6** is not "deaminagenic" since it lacks an enamine functionality.

Our kinetic studies with the WT enzyme show that SAM stabilizes the binding of the enzyme to *Eco*RII site by about 14-fold. This value was determined by comparing K_m^{DNA} values of two reactions that proceed in the absence (^{3}H exchange) and the presence (methyl transfer) of SAM. These results are consistent with our finding that SAM and SAH stimulates the stable and specific binding of M.*Eco*RII to its recognition site (Wyszynski et al., 1993) and the finding with M.*Hae*III that the binding of the C71A mutant to DNA is stimulated by SAM (Chen et al., 1993). In contrast, K_m^{DNA} values of methyl transfer and ^{3}H release reaction obtained for M.*Hha*I were comparable, 2.3 and 2.1 nM, respectively (Wu & Santi, 1987), suggesting that M.*Hha*I, unlike M.*Eco*RII, binds equally well in the absence and in the presence of SAM.

We have previously reported the results of a genetic assay that suggested that C186S mutant can efficiently form covalent complexes with azaC DNA (Wyszynski et al., 1992). The assay used the sensitivity of certain repair-deficient strains of *E. coli* containing a C5 MTase to 5-azacytidine, as a measure of the complex formation. It was found that *E. coli* cells expressing C186S mutant were only 10 times less sensitive to killing by 5-azacytidine than cells containing the WT enzyme (Wyszynski et al., 1992). This was surprising in view of the high pK_a of the hydroxyl group in free serine.

The *in vitro* studies reported here confirm the suspected (Wyszynski et al., 1992) reactivity of C186S toward azaC DNA. C186S mutant methylates azaC DNA at a much faster rate than normal DNA. When the same ratio of enzyme to substrate was used, duplex VI was methylated 2400 times faster than than duplex III (Figure 6). This result explains the previous genetic observation that cells containing this mutant are sensitive to 5-azacytidine (Wyszynski et al., 1992) and shows that in some situations this mutant can be quite reactive. It also points out that a C5 MTase in which the catalytic cysteine is replaced with serine should not be treated as a null mutant.

The reason for the reactivity of the serine mutant toward azaC DNA can be explained in terms of the chemistry of 5-azacytosine. The increased electronegativity at position 5 resulting from the replacement of carbon-5 by nitrogen should polarize carbon-6 making it more electropositive. As a result, C-6 in 5-azacytidine should be much more reactive toward weak nucleophiles such as hydroxyls. We conclude that the higher reactivity of azaC DNA must assist the mutant enzyme in catalysis.

In summary, the results presented here have led to three important conclusions regarding the structure and reaction mechanism of C5 MTases. First, our results strongly suggest that the catalytic cysteine is activated by a nearby base and this basic residue is likely to be a histidine in many C5 MTases. Second, we have shown that 5-azacytosine in DNA

is much more reactive than cytosine toward attacking nucleophiles. For this reason, experiments involving cells that are grown in the presence of 5-azacytosine should be interpreted with caution. It is likely that proteins and small molecules other than C5 MTases will react with azaC DNA. Finally, we have pointed out that SAH inhibits M.*Eco*RII-mediated 5-H exchange, methyl transfer, and cytosine deamination. To explain this observation, we have presented a unified reaction scheme that integrates the mechanisms of all the three reactions. This scheme provides a more integrated view of the action of C5 MTases than presented before.

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