620–627 Nucleic Acids Research, 1995, Vol. 23, No. 4

Functional analysis of Gln-237 mutants of *Hha*I methyltransferase

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Received October 27, 1994, Revised and Accepted January 6, 1995

ABSTRACT

When the *Hha*I (cytosine-5) methyltransferase (M.*Hha*I) binds DNA it causes the target cytosine to be flipped 180° out of the helix. The space becomes occupied by two amino acids, Ser-87 and Gln-237, which enter the helix from opposite sides and form a hydrogen bond to each other. Gln-237 may be involved in specific sequence recognition since it forms three hydrogen bonds to the orphan guanosine, which is the partner of the target cytosine. We have prepared all 19 mutants of Gln-237 and tested their biochemical properties. We find that mutations of this residue greatly affect the stability of the M.*Hha*I–DNA complex without affecting the enzyme's specificity for the target sequence. Surprisingly, all mutants retain detectable levels of enzymatic activity.

INTRODUCTION

The function of 5-methylcytosine DNA methyltransferases (m5C-MTases) is to transfer a methyl group from S-adenosylmethionine (AdoMet) to the C5 position of a cytosine residue contained within a specific double-stranded DNA sequence. Methylated DNA is involved in many different biological processes in organisms ranging from bacteria to mammals. One common role in bacteria is the involvement of 5-methylcytosine (m5C) in protecting host DNA from the action of restriction enzymes (1).

All m5C-MTases share a common architecture (2,3) composed of 10 conserved motifs. There are six highly conserved (I, IV, VI, VIII, IX and X) and four moderately conserved motifs (3). A variable region, localized between motifs VIII and IX, is involved in both DNA sequence recognition and base choice for methylation (4–6). Motif I is required for binding the co-factor AdoMet (7) and motif IV, the PC motif, contains the catalytic center (8). The mechanism employed by m5C-MTases has been studied extensively and the key residue in the PC motif is a conserved cysteine, whose thiol group is involved in nucleophilic attack to form a covalent bond with the target cytosine This activates the 5-position of cytosine, which picks up the methyl group from AdoMet to form a dihydrocytosine intermediate (8–12). Subsequent β-elimination of the proton at position 5 releases the enzyme with the formation of 5-methylcytosine in the DNA. In the process AdoMet is converted to S-adenosylhomocysteine (AdoHcy).

The *Hha*I methyltransferase (M.*Hha*I), has been studied extensively and serves as a paradigm for the m5C-methyltransferases (reviewed in 13). Structures are available for M.*Hha*I complexed with AdoMet (14) and for a co-crystal formed between M.*Hha*I, AdoHcy and a duplex oligonucleotide target (15). Analysis of these structures suggests a novel mechanism for methyltransferase action whereby the cytosine targeted for methylation is flipped completely out of the DNA helix without disturbing the rest of the DNA structure. In the process, the enzyme undergoes conformational changes in two regions The catalytic motif moves nearly 25 Å toward the DNA minor groove, and the variable region shifts such that Gln-237 in one of the recognition loops forms three hydrogen bonds with the orphan guanine base that is the normal base-pairing partner of the target cytosine. It also forms one hydrogen bond with Ser-87 located in the catalytic motif IV (14,15). The two loops containing Gln-237 and Ser-87 appear crucial to DNA binding since they form two fingers that grasp the DNA at the position of the target G–C base pair and actually infiltrate into the DNA appearing to hold open the position occupied by the target cytosine (Fig. 1). To assess the importance of Gln-237 in the catalytic reaction we have undertaken site-directed mutagenesis of this residue.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K-12 strain ER1727 [Δ(*mcrC-mrr*)*102*::Tn10, *mcrA1272*::Tn10/F′lac *proA*$^+$*B*$^+$ *lacI*^{q}/Δ(*lacZ*)*M15*] and strain ER1969 [McrBC$^+$] were kindly provided by E. Raleigh Plasmid pUHE-25, containing a T7 promoter, was kindly provided by U. Deuschle. Plasmid pHSH0-1 contains a mutant form (Ile to Leu substitution at position 2) of M.*Hha*I expressed from a T7 promoter in pUHE-25 (4). The properties of this mutant are indistinguishable from the wild-type enzyme (4) Plasmid pGEM-3Zf(+) (Promega) was used for preparation of single-stranded DNA for site-directed mutagenesis.

Enzyme and reagents

[^{3}H-Me]-AdoMet (77 Ci/mmole), deoxycytidine-5′[α-^{32}P]-triphosphate and deoxyadenosine-5′[α-^{32}P]-triphosphate (3000 Ci/mmole) were purchased from Amersham (Arlington. IL)

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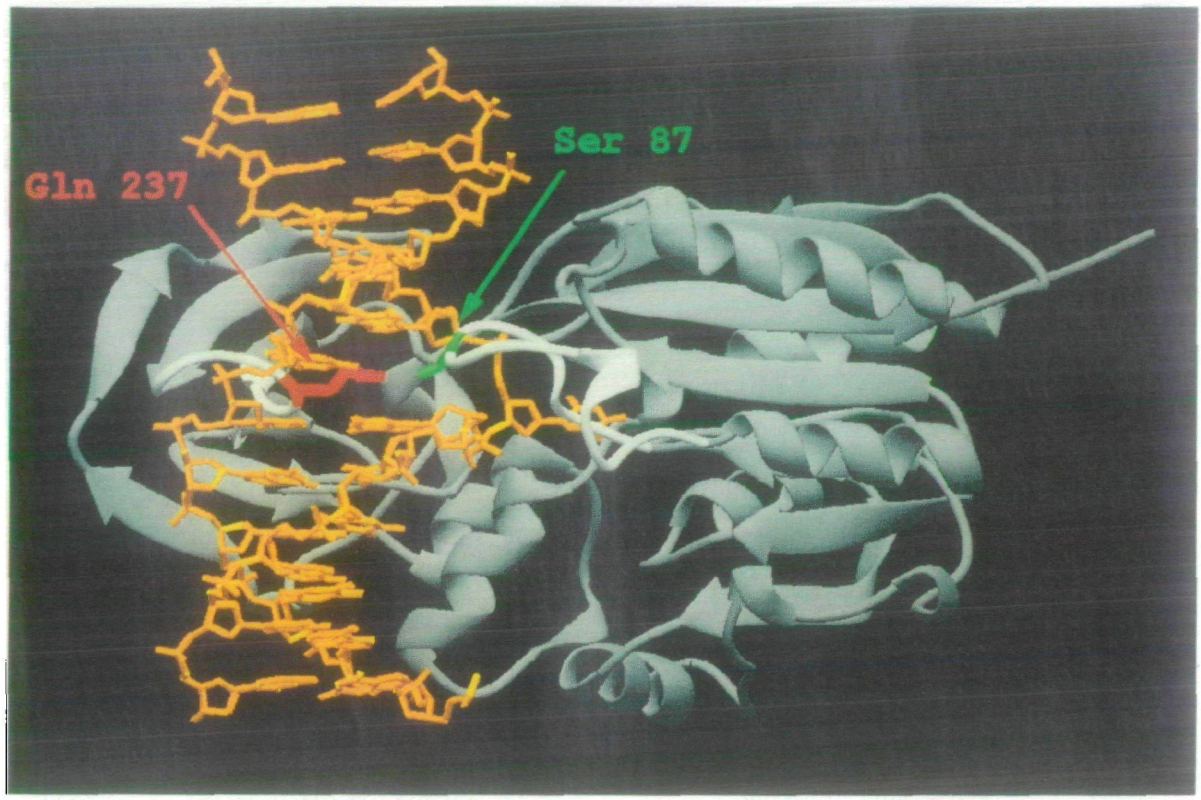

Figure 1. Structure of M.*Hha*I binding to DNA. The two loops (233–240 and 80–99) that infiltrate the helix are shown in white. Gln-237 is shown in red and Ser-87 is shown in green.

Restriction enzymes, T4 DNA ligase, Vent DNA polymerase and bacteriophage lambda DNA were from New England Biolabs and were used according to the manufacturer's specifications. DNA sequencing was performed using a kit from United States Biotechnology and site-directed mutagenesis was performed using a kit from Amersham. A double primer-based mutagenesis kit was purchased from Clontech. Antibodies directed against M.*Hha*I were prepared in rabbits by Cocalico Biologicals using purified enzyme prepared as described (16).

Oligonucleotides

Oligonucleotides 1–12 were synthesized by the Cold Spring Harbor Oligonucleotide Core Facility. Oligonucleotide 13 was from New England Biolabs.

1. GTAGGAAAAGGTGGGNNNGGAGAACGAATTTA
2. GTAGGAAAAGGTGGGGATGGAGAACGAATTTA (Asp)
3. GTAGGAAAAGGTGGGAAAGGAGAACGAATTTA (Lys)
4. GTAGGAAAAGGTGGGAATGGAGAACGAATTTA (Asn)
5. GTAGGAAAAGGTGGGTTTGGAGAACGAATTTA (Phe)
6. GTAGGAAAAGGTGGGATGGGAGAACGAATTTA (Met)
7. GTAGGAAAAGGTGGGGGAGGAGAACGAATTTA (Gly)
8. GTAGGAAAAGGTGGGATCGGAGAACGAATTTA (Ile)
9. GTAGGAAAAGGTGGGTACGGAGAACGAATTTA (Tyr)
10. ATAGCTGCCTCAAAAGCATGCTTAGCTGACTTGGACTC
11. TCGGATGTTGTGGGTCAGCGCCTGATACTGTACCAGT
12. GACTGGTACAGTATCAGGCGCTGACCCACAACATCCG
13. TCGGATGTTGTGGGTCAGFGCCTGATACTGTACCAGT

Oligonucleotides 1–9 were used for mutagenizing Gln-237. Oligonucleotides 11–13 were used for the methyltransferase binding assays. Oligonucleotide 13 is identical to 11 except that it contains 5-fluorocytosine at the target (underlined).

Mutagenesis of methyltransferases

The Asp, Lys, Asn, Phe, Met, Gly, Ile and Tyr mutants were generated by site-directed mutagenesis (Amersham kit) with oligonucleotides 2–9 (17). Oligonucleotide 1 is a degenerate primer (NNN) from which M.*Hha*I mutants containing codons encoding Ala (GCT), Cys (TGC), Glu (GAA), His (CAC), Leu (TTG), Pro (CCA), Arg (CGC), Ser (AGC), Thr (ACG), Val (GTG) and Trp (TGG) were isolated. The mutants were generated by double primer mutagenesis (Clontech kit) with the following modifications. Degenerate oligonucleotide 1 was used to mutate the M.*Hha*I gene and oligonucleotide 10 used to destroy a *Hin*dIII restriction site in the plasmid. Non-mutagenized plasmids can therefore be removed by digestion with *Hin*dIII prior to transformation. Oligonucleotide 10 also contains a new *Sph*I site providing a diagnostic restriction assay for mutagenized plasmids. The plasmid pHSH0-1 was denatured and annealed with primers 1 and 10 at 100°C for 5 min, then chilled in an ice water bath for 5 min. Primed DNA was used to synthesize the daughter strand in the presence of dNTP, T4 DNA polymerase and T4 DNA ligase. Synthesized DNA was then methylated with *dam* methyltransferase (to prevent repair of the daughter DNA strand after transformation into strain ER1727) and digested by *Hin*dIII. Digested DNA was precipitated and transformed into ER1727 cells. The transformants were pooled and plasmid DNA was

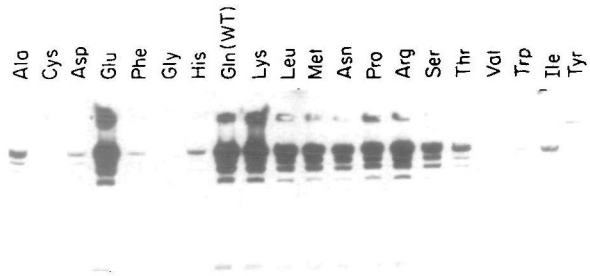

Figure 2. Mutant methyltransferase activities. The mutant methyltransferases were incubated with *Hin*dIII-digested phage λ DNA in the presence of [^{3}H]-AdoMet at 37°C for 1 h as described in Materials and Methods. Methylated DNA was separated on a 0.8% agarose gel and autoradiographed. Shown is an over-exposure to detect weak methylation events.

prepared. Plasmid DNA was again cut by *Hin*dIII and re-transformed into ER1727 cells. Plasmids that lost a *Hin*dIII site and acquired an *Sph*I site were sequenced to determine if the DNA insert was mutated.

Small scale protein purification for methyltransferase assays

Mutant methyltransferase proteins, used for the experiments of Table 1 and Figures 2 and 3, were purified by immunoprecipitation. Overnight cultures containing plasmids expressing the M.*Hha*I mutants were diluted 200-fold into 5 ml LB medium. Expression of the proteins was induced by adjusting the culture to 400 μM IPTG at an OD_{600} of 0.5. After 2 h induction, the cells were harvested by centrifugation. The cells were then lysed in 2 × lysis buffer (100 mM Tris–HCl, pH 7.4, 300 mM NaCl, 20 mM Na_2EDTA, 10% glycerol and 2 mM PMSF). The cells were frozen and thawed four times and sonicated briefly. Lysed cells were centrifuged for 10 min at 14 000 *g*. The supernatants were collected and incubated with rabbit anti-M.*Hha*I antibody for 1 h at 4°C and protein-A beads added for an additional incubation of 1 h at 4°C. The immunoprecipitated proteins were washed three times with washing buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 100 mM Na_2EDTA, 10% glycerol and 2 mM PMSF). The levels of mutant proteins were quantitated by Western blotting. The beads containing M.*Hha*I mutant proteins were stored at 4°C and used directly for assays of methyltransferase activity.

Large scale protein purification for kinetic studies

Cells were grown and induced for expression of M.*Hha*I mutant proteins as described above. Pelleted cells were washed with Hepes buffer (10 mM Hepes, pH 7.2, 5 mM Na_2EDTA, 10% glycerol, 0.1% β-mercaptoethanol). The cells were then incubated in the presence of lysozyme (50 μg/ml) in Hepes buffer on ice for 45 min, frozen and thawed four times and sonicated briefly. Following centrifugation the pellets, which contained the methyltransferase proteins, were washed twice in Hepes buffer and the proteins extracted by resuspending in phosphate buffer (KPO_4, pH 7.2, 0.4 mM NaCl, 5 mM Na_2EDTA, 10% glycerol, 2 mM PMSF, 0.1% β-mercaptoethanol). Nucleic acids were removed by precipitation with 5 mg/ml protamine sulfate. The precipitates were removed by centrifugation and the supernatants, which contained the M.*Hha*I mutant proteins were purified using a Pharmacia MONO-S FPLC column (16,17). Analysis on a Coomassie stained SDS–PAGE gel indicated that each M.*Hha*I mutant protein was purified to a single band. AdoMet-free methyltransferases were obtained by dialyzing for 78 h in a buffer containing 10 nM [^{3}H]-AdoMet and then against a buffer containing 10 mM $KHPO_4$, pH 7.25, 10 mM Na_2EDTA and 100 mM NaCl at 16°C with the buffer changed every 6 h. Dialysis was stopped when the [^{3}H]-AdoMet counts for the samples and the dialysis buffer reached background levels.

DNA methylation

Two methods were used to determine the enzymatic activity of the methyltransferases. First, M.*Hha*I mutant proteins (100 nM), purified by immunoprecipitation, were incubated with *Hin*dIII-digested lambda DNA (4 μM) in the presence of [^{3}H]-AdoMet (1.3 μM) at 37°C for 1 h. The reactions were divided into two fractions for quantitation of the incorporated [^{3}H]-label. One fraction was separated in a 0.8% agarose gel and the DNA concentrations first determined to be similar by staining with ethidium bromide. The gel was then treated with ^{3}H autoradiography enhancer (Amersham), dried, and exposed to X-ray film. The other fraction was quantitated using a DE-81 filter binding assay as described previously (5).

Methyltransferase activities were also determined by testing for $McrBC^+$ restriction (18). Cells harboring plasmids for expression of the M.*Hha*I mutant genes were grown to an OD_{600} of 0.5 and induced with 400 μM IPTG for 2 h. The plasmids were then isolated and transformed into $McrBC^+$ (ER1969) or $McrBC^-$ (ER1727) *E.coli* cells. The number of transformants obtained in an $McrBC^+$ line was compared to that obtained in an $McrBC^-$ line. Methylated plasmid DNA is restricted in $McrBC^+$ strains.

Methyltransferase specificity

Methyltransferase specificity was determined in a 20 μl reaction by incubating the methyltransferase proteins (100 nM) in the presence of [^{3}H]-AdoMet (1.3 μM), with non-methylated plasmid DNA (pUHE-25) (150 nM) or with plasmid DNA (pHSHO-1) (150 nM) pre-methylated by M.*Hha*I *in vivo*. The radiolabeled DNA was quantitated by binding onto DE-81 paper. Incorporation of any radioactivity into pre-methylated plasmid DNA is indicative of a loss of specificity.

Gel shift DNA binding assay

M.*Hha*I mutant proteins either pure or immunoprecipitates (100 nM) were incubated, in the presence of 10 μM AdoHcy, with 100 nM of a ^{32}P-labeled 37mer duplex (oligonucleotides 11 and 12) containing a single GCGC site using a protocol described previously (19). The reactions were incubated at 22°C for 1 h, then loaded onto a 10% native polyacrylamide gel as described (19) and fractionated at 120 V at 4°C for 2 h. The gel was dried and quantitated by phosphorimager analysis. The percentage of binding of the mutant methyltransferase to the duplex was determined relative to binding of the wild-type methyltransferase.

Oligonucleotides that contain 5-fluorocytosine at the target site form an irreversible covalent complex with m5C-methyltransferases (10). We used this as the basis for determining the specific activity of the purified methyltransferases for the binding of DNA. To form a covalently trapped ternary complex, 100 nM of the Glu-237 mutant or wild-type *Hha*I methyltransferase was

incubated with an excess (60 μM) of duplex DNA containing the fluorinated oligonucleotide 13 in the presence of 50 μM AdoMet. The reaction was incubated at 16°C for 2 days or 22°C for 1 day and the covalent ternary complex detected on a 9% SDS–PAGE gel by Coomassie staining.

Measurement of kinetic constants

Apparent K_m's for DNA and AdoMet as well as k_{cat} values were determined by monitoring [^{3}H]-methyl transfer from [^{3}H]-AdoMet to a 37mer DNA duplex (oligonucleotides 11 and 12). Each 20 μl reaction mixture contained 50 mM Tris–HCl, pH 7.4, 10 mM Na_2EDTA, 10 mM β-mercaptoethanol, 200 μg/ml bovine serum albumin (BSA) and 0.05–1 nM wild-type or mutant methyltransferase. Both the DNA and AdoMet concentrations were varied from 1 to 100 nM. The [^{3}H]-AdoMet was added to a final concentration of 1 3 μM. The reaction was linear for at least 30 min. The final product of the reaction, AdoHcy, never accounted for more than 0.1% of the input AdoMet. The reaction was stopped after a 10 min incubation at 37°C and samples were spotted onto DE-81 filters. Following washing five times with 0.2 M $(NH_4)_2CO_3$, twice with H_2O and once with 100% ethanol, the filters were dried and quantitated by liquid scintillation counting. The kinetic parameters $K_{m(DNA)}$ and V_{max} were calculated from a plot of 1/V versus 1/[S] (Lineweaver–Burk) using the 'Enzyme Kinetics' program (D. G. Gilbert Software). k_{cat} for wild type or mutant methyltransferases was calculated using M_r = 37 000 for M.*Hha*I (12). It should be noted that M.*Hha*I concentrations used in all calculations were total M.*Hha*I wild type or mutant proteins.

RESULTS

Activities of mutant methyltransferases

Site-directed mutagenesis was used to replace Gln-237 with all 19 other amino acids and the proteins were purified by immunoprecipitation and used for the experiments in Table 1 and Figures 2 and 3. In control experiments (not shown) we found that equivalent amounts of pure M.*Hha*I or immunoprecipitated M.*Hha*I gave equivalent results in terms of DNA shifted in the standard assay. However, the bands representing the protein–DNA complexes had significantly different mobilities with the pure M.*Hha*I–DNA complex running much faster. We interpret this to mean that M.*Hha*I did not dissociate from the immunoprecipitate.

Table 1. Properties of wild type and mutant M *Hha*I

Mutant	[^{3}H] / λ DNA filter assay	DNA binding (GCGC 37 oligomer)	Methylase specificity (c p m) premethylated / non–methylated DNA	DNA	Number of transformants McrBC$^+$ / McrBC (colonies)
Vector	0 1	0 0	81 / 79	(0 2)	51 / 49
Gln (wt)	100 0	100 0	72 / 31455	(100 0)	0 / 61
Ala	19 0	0 4	73 / 6140	(19 0)	0 / 30
Arg	42 0	0 9	72 / 19832	(63 0)	0 / 37
Asn	30 0	0 9	70 / 13212	(43 0)	0 / 36
Asp	3 0	0 2	75 / 943	(2 8)	0 / 27
Cys	2 0	0 2	52 / 629	(2 7)	0 / 90
Glu	64 0	1 0	71 / 20128	(65 0)	0 / 42
Gly	2 0	0 1	51 / 601	(2 6)	0 / 27
His	16.0	0 2	47 / 5021	(24 0)	0 / 19
Ile	3 0	0.1	71 / 802	(2 5)	0 / 97
Leu	50.0	1 3	56 / 12386	(51 0)	0 / 34
Lys	68 0	2 0	74 / 22322	(69 0)	0 / 33
Met	39.0	0 8	72 / 10121	(32 0)	0 / 80
Phe	3.5	0 1	63 / 1100	(3.9)	0 / 31
Pro	29 0	0 2	80 / 10488	(30 0)	0 / 41
Ser	25 0	1 2	41 / 7921	(44 0)	0 / 52
Thr	14.0	0.1	68 / 4103	(13.0)	0 / 44
Trp	2.5	0 1	68 / 792	(2 7)	0 / 33
Tyr	1 0	0 1	62 / 489	(1 8)	0 / 88
Val	1.5	0 1	69 / 412	(1 3)	0 / 22

The values shown in columns 2 and 3 are percentages normalized against the wild type M.*Hha*I (Gln) In column 4, the raw numbers are the average of two determinations and the numbers in parentheses show values normalized against the wild type ratios (c p.m. incorporated into non–methylated DNA/c p m incorporated into premethylated DNA) to allow comparison with the data in column 2

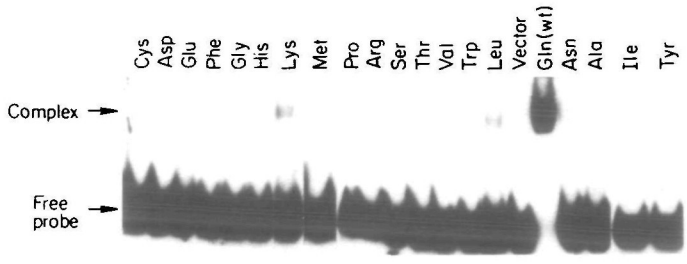

Figure 3. Binding of mutant methyltransferases to DNA. The target DNA is a 37mer oligonucleotide containing a single GCGC binding site. Mutant methyltransferases, including the wild type, were purified by immunoprecipitation and quantitated by Western analysis. The lane marked 'vector' is an immunoprecipitate prepared from cells containing the vector alone. Approximately equal amounts (100 nM) of each methyltransferase and ^{32}P-labeled oligomer were incubated with AdoHcy (10 μM) in a standard binding reaction (see Materials and Methods).

Enzymatic activity of the methyltransferases was tested using both a filter binding assay and McrBC$^+$ restriction. The mutants were tested for their inherent methyltransferase activity using an unmodified substrate DNA. As shown in Table 1, column 2, all substitutions diminished, but did not abolish, enzyme activity when compared to the wild-type. To check the significance of the low counts resulting from some of the mutants in the filter binding assay the methylated DNA was also analyzed by agarose gel electrophoresis. Figure 2 shows an over-exposure of a film to detect weakly methylated fragments. The agarose gel assays and the results from the filter binding assay were in agreement and confirmed that the low counts observed in some mutants were due to methylation of the DNA and not just to some trapping artifact. The mutant enzymes were also shown to be active by an McrBC$^+$ restriction assay. McrBC$^+$ strains can restrict DNA methylated by M.*Hha*I. Plasmid DNAs were prepared from cells carrying either the wild-type or mutant M.*Hha*I genes, which had been treated with IPTG to induce methyltransferase expression for *in vivo* methylation of the plasmid. The DNA was then re-transformed into an McrBC$^+$ (strain ER1969) or an McrBC$^-$ cell line (strain ER1727) as control. The transformation efficiency from the two cell lines were compared and the results are shown in Table 1. The control vector DNA, carrying no methyltransferase gene, was not restricted in either cell line. However, plasmids carrying wild-type or mutant methyltransferase genes were completely restricted in McrBC$^+$ cells, but not in McrBC$^-$ cells. This indicates that the mutant methyltransferases are all enzymatically active *in vivo*.

Target specificity of mutant methyltransferases

Plasmids that have been pre-methylated *in vivo* by a specific enzyme cannot be further methylated *in vitro* by the same enzyme. We took advantage of this as an assay for specificity. As shown in Table 1, column 4, plasmids isolated from cells expressing wild-type M.*Hha*I are no longer substrates for further introduction of [^{3}H]-methyl groups by the mutant or wild-type methyltransferases. However, wild-type or mutant methyltransferases were able to methylate control vector plasmid or λ DNA that was not previously methylated. These results indicate that the mutant methyltransferases are not altered in their target specificity.

Binding activities of mutant methyltransferases

Since Gln-237 is involved in hydrogen bonding to the orphan guanine base that is the partner of the methylated cytosine, it is possible that the diminished enzymatic activities of the mutant methyltransferases reflect the decreased ability of the enzymes to bind DNA. To assess the ability of each enzyme to bind DNA, mobility shift assays were carried out. In control experiments (not shown) no binding could be detected to a non-specific oligonucleotide. Using an oligonucleotide containing the recognition sequence binding experiments gave the results shown in Figure 3 and summarized in Table 1. All mutants are significantly diminished in their ability to form stable DNA–protein complexes, showing only 0.1–2% of the binding activity of the wild-type enzyme. Figure 3 shows an over-exposed film to detect weak binding. The binding activities, as quantitated by phosphorimager analysis, are shown in Table 1. Attempts were made to obtain $K_{d(DNA)}$ values using several of the FPLC purified mutant proteins (Ala, Glu, His, Leu, Ser and wt Gln). The gel shift assay, however, was not sensitive enough to measure the increase in $K_{d(DNA)}$. Similar to earlier observations of others with M.*Msp*I (19), attempts to use high concentrations of enzymes and DNA in this assay were not successful and led to artifactual smearing and non-specific complex formation. To determine if the mutant methyltransferase proteins used in the gel-shift assays were active, covalent-trap experiments were performed. If the methyltransferase is functional, then on prolonged incubations with oligonucleotides containing a fluorinated cytosine in the target sequence it should be trapped as an irreversible covalent ternary complex. This complex can be readily identified by SDS–PAGE analysis. Using oligonucleotides 12 and 13 in such an experiment, it can be seen that 50% of a preparation of the Glu-237 mutant protein formed the covalent ternary complex as compared with 80% for the wild-type protein (Fig. 4A). However, as shown in Figure 4B, an identical amount of the Glu-237 mutant protein results in only about 2% of the protein forming a complex with normal target DNA (oligonucleotides 11 and 12). This experiment therefore indicates that the $K_{d(DNA)}$ value for the Glu-237 mutant is at least 50-fold greater than the wild-type $K_{d(DNA)}$ value of 1.2 nM (17).

Steady state kinetic analysis

Since we were unable to obtain $K_{d(DNA)}$ values for most of the mutant methyltransferases, we decided to measure their kinetic constants, $K_{m(DNA)}$ and k_{cat}. The wild-type and five mutant methyltransferases were purified to homogeneity and their $K_{m(DNA)}$ and V_{max} determined, as described in Materials and Methods. Figure 5 shows the Lineweaver–Burk plot from which $K_{m(DNA)}$ and V_{max} for wild type M.*Hha*I and the Glu-237 mutant were calculated. The results are shown in Table 2. Compared to the wild-type enzyme, the Ala-237 mutant exhibited a similar $K_{m(DNA)}$, with a 9-fold decrease in the k_{cat}. $K_{m(DNA)}$ values for the Glu, Leu, His and Ser mutants were increased 2–3-fold, and their k_{cat} values decreased 2–16-fold. In comparison to the wild-type enzyme, the catalytic efficiencies of the mutants ($k_{cat}/K_{m(DNA)}$) were reduced 4–28-fold. $K_{m(AdoMet)}$ values remained at ~20 nM.

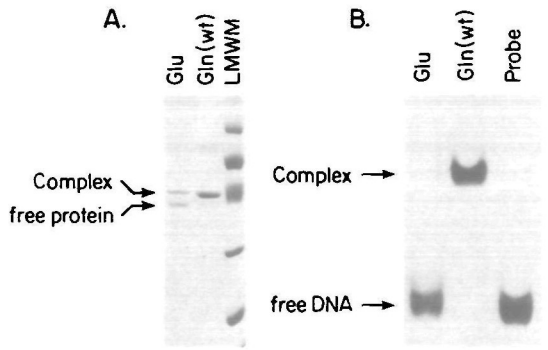

Figure 4. DNA–protein complex formation with DNA containing 5-fluorocytosine. The Glu-237 mutant or wild-type Gln-237 M.*Hha*I was purified on an FPLC mono-S column as described in Materials and Methods. (**A**) 100 nM protein was incubated with an excess of 60 μM fluorinated oligomer (oligonucleotides 12 and 13) and AdoMet. The trapped covalent DNA–protein complex was separated from protein by SDS–PAGE and stained with Coomassie Blue. (**B**) 100 nM protein was incubated with 100 nM ^{32}P-labelled oligomer (oligonucleotides 11 and 12). Protein bound DNA was separated from free oligonucleotide on a 10% native polyacrylamide gel.

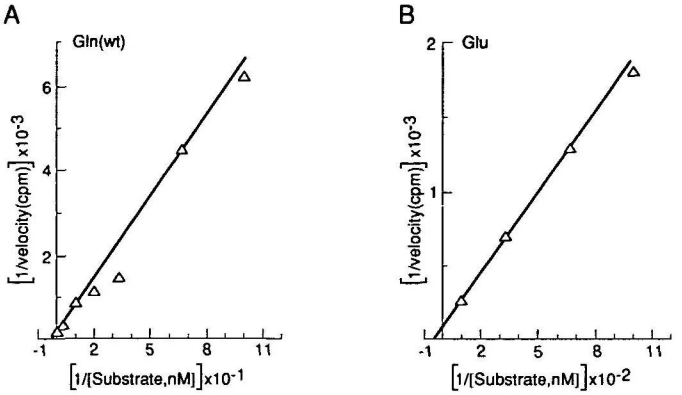

Figure 5. Kinetics of methylation by Gln-237 (wt) and the Glu-237 mutant of M.*Hha*I. Reactions contained the the indicated concentrations of substrate oligonucleotides (11 and 12). The enzyme concentration was 0.05 nM in (**A**) and 1 nM in (**B**). [^{3}H]-AdoMet (77 Ci/mmole) was used at a final concentration of 1.3 μM.

Table 2. Catalytic and substrate binding parameters for wild type and mutant M.*Hha*I

Methylase	K_m[DNA] (nM)	K_m[SAM] (nM)	V_{max} (nmol·min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (10^5 M^{-1} S^{-1})
Ala	81	17	9.5	0.35	0.70
Glu	221	24	40.0	1.50	1.10
His	141	18	5.9	0.20	0.30
Leu	120	24	38.0	1.40	1.90
Ser	133	17	6.0	0.23	0.28
Gln (wt)	69	15	87.0	3.20	7.70

The kinetic constants were determined from a minimum of five repetitions of each experiment and the variation was from 11 to 24%.

DISCUSSION

The lower enzymatic activity of the mutant methyltransferases may be due to the formation of DNA–protein complexes which are less stable than those formed by the wild-type enzyme since the hydrogen bonds formed by Gln-237 may help to stabilize the DNA–protein complex and to complete the methylation reaction. If this is the case we should expect formation of unstable complexes to be reflected in increased values for the dissociation constant, $K_{d(DNA)}$. We first determined if a complex between mutant methyltransferases and DNA can be formed. We partially purified 19 mutant methyltransferases by immunoprecipitation to determine their DNA binding properties using a mobility shift assay. Under the normal experimental conditions for measurement of the wild-type enzyme's binding property, we failed to detect significant DNA–protein complexes for any of the mutant methyltransferases. Since non-specific interactions predominate at high enzyme concentrations for the gel-shift assays, we used low concentrations of enzymes whereupon a weak, but detectable gel-shift was apparent. Covalent trap experiments were carried out to determine the degree of activity of the five mutant proteins (Ala, Glu, His, Leu, Ser). Using the Glu-237 mutant and wild-type enzyme as examples, we found that minimally 50 and 80% of the proteins can form the irreversible ternary complex, as shown in Figure 4A. Thus the loss of binding activity observed in the gel shift assay cannot be simply attributed to inactive enzymes, but rather suggests that the $K_{d(DNA)}$ values for the mutant enzymes are much greater than that of the wild-type enzyme.

Given the limitations of the gel shift assay for analysis of the mutant proteins, we decided to measure the $K_{m(DNA)}$ and k_{cat} for the mutant methyltransferases directly and calculated the catalytic efficiency $k_{cat}/K_{m(DNA)}$. It was found that neither the $K_{m(DNA)}$ nor the k_{cat} of the mutant methyltransferases were markedly different from the wild-type enzyme. The $K_{m(DNA)}$ increased up to 3-fold and the k_{cat} decreased 2-fold in the case of the Glu-237 and Leu-237 mutants and decreased by 9-, 16- and 14-fold in the Ala-237, His-237 and Ser-237 mutants, respectively. These substitutions all resulted in a 4–28-fold decrease in the catalytic efficiency of the enzymes. These changes were not due to an alteration of the $K_{m(AdoMet)}$ which remained similar to that of the wild-type enzyme at about 20 nM. These results indicate that Gln-237 and the unique hydrogen bonds it provides are not essential for catalysis. Furthermore, the mutants do not give rise to a change of DNA recognition specificity. These data may also explain why Gln-237 is not conserved among the m5C-methyltransferases since all substitutions show some degree of activity. Other groups had previously carried out kinetic studies with M.*Hha*I (12,16). When compared with our data, there was a 2-fold difference in the k_{cat} values and a 30-fold difference in the $K_{m(DNA)}$ values. These differences probably result from the use of different DNA substrates as well as different methodologies.

It is surprising that all 19 mutants at Gln-237 continue to show methyltransferase activity, albeit at reduced levels. Examination of the structure (Fig. 6) shows that only one of the four hydrogen bonds involving Gln-237 would be universally retained in all the mutants—that between the main chain carbonyl and O6 of guanine, although the precise geometry of that interaction might change. Two hydrogen bonds, between the carbonyl oxygen of

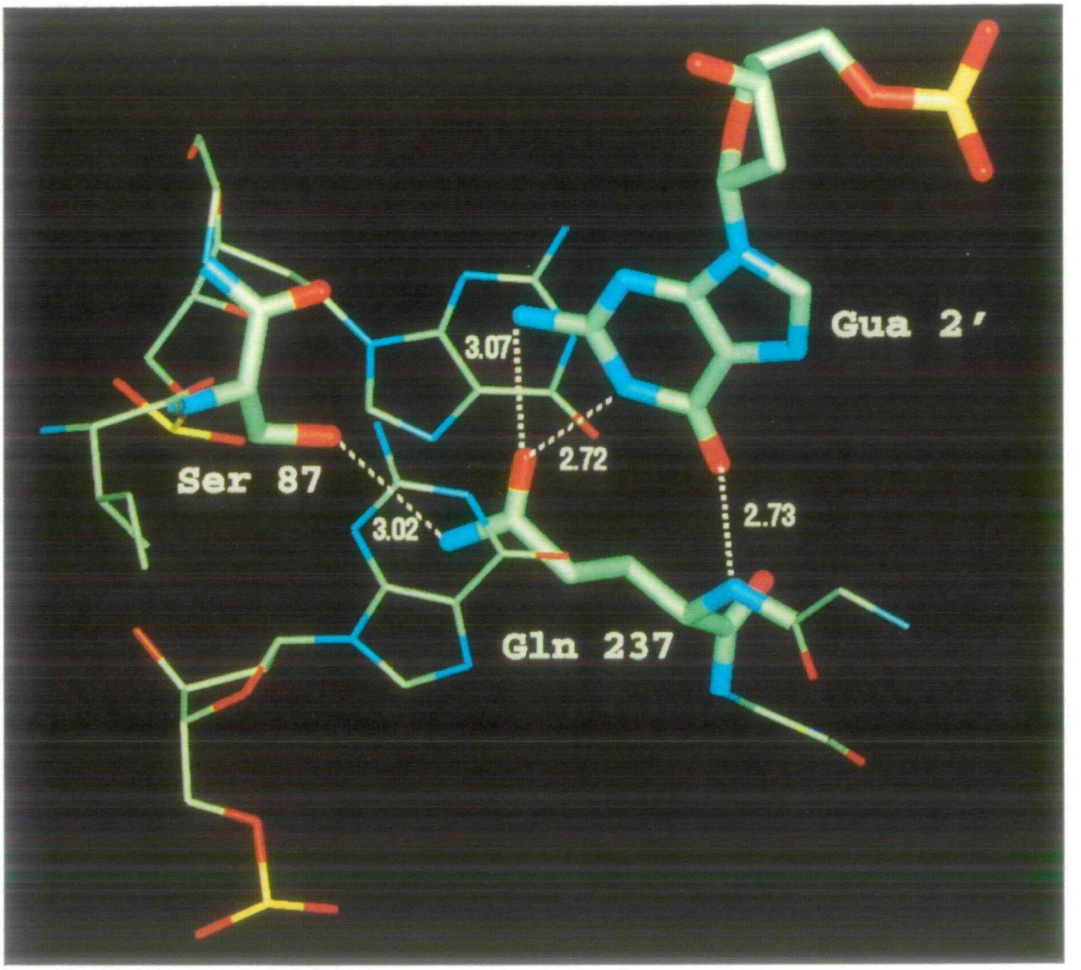

Figure 6. Close up of the interaction between Gln-237, the orphan guanosine residue and surrounding amino acids. Key residues are shown as heavy lines. The dashed lines show hydrogen bonds. One is between the side chain oxygen of Ser-87 to the side chain nitrogen of Gln-237 (3.02 Å). Two form between the side chain carbonyl group of Gln-237 and N1 (2.72 Å) and the amine on C2 (3.07 Å) of the orphan guanine. One lies between the main chain amide of Gln-237 and O6 (3.02 Å) of the orphan guanine.

the side chain of Gln-237 and both the ring nitrogen (N1) and the primary amine at C2 of the orphan guanosine, are lost with most side chain substitutions. Additionally, the fourth hydrogen bond between the nitrogen of the side-chain amide in Gln-237 and the hydroxyl group of Ser-87 could not form. The disruption of these hydrogen bonds could be expected to lower the half-life of the catalytically-active state in which the target cytosine (Cyt-2) is flipped out. As observed we would expect this effect to be most pronounced in the gel shift assays which measure DNA binding directly. There was no apparent correlation between the polar or non-polar nature of the side chains and enzymatic activity.

In summary, it seems that Gln-237 is less important than might have been thought from its hydrogen bonding pattern. Differences in methylation efficiencies by these enzymes can all be accounted for by the effects of the mutations on the stability of the protein–DNA complex. Our results argue that Gln-237 is not essential for either recognition of the orphan guanosine, the initial flipping of the target cytosine or for holding the helix open while catalysis takes place. Furthermore, the hydrogen bond between Gln-237 and its partner Ser-87 appear to be a peculiarity of the M.*Hha*I system that adds stability, but is not required. Similarly the hydrogen bonds between the amide side chain of Gln-237 and the orphan guanosine may be fortuitous and useful only to stabilize the structure after base eviction. In different m5C-methyltransferases other interactions, perhaps hydrophobic ones, can be expected since the most common homolog of Ser-87 is an alanine residue (13). Because bulky residues such as glutamic acid and lysine can substitute for Gln-237 and still permit catalysis when paired with Ser-87 it is possible that size is more important than a specific side-chain. The ability of the side chain to fill the space successfully may be the most important aspect of the residue occupying position 237. It will be interesting to see whether there is a preferred partner for alanine in other m5C-methyltransferases.

ACKNOWLEDGEMENTS

We thank Jeff Kuret and Saulius Klimasauskas for discussions and Karen McCloy for technical assistance. We gratefully acknowledge the help of Sanjay Kumar in preparing Figures 1 and 6 and for critical comments on the manuscript. We also thank Shuang Xu, Ira Schildkraut and Geoff Wilson for useful comments on the manuscript. SM is the recipient of an American Cancer Society Fellowship. This work was supported by grants

from the NSF (DMB-8917650) and NIH (GM46127). Centralized facility support for oligonucleotide synthesis was from a Cancer Center Support grant from the NCI (CA45508).

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