Mutational analysis of conserved residues in *Hha*I DNA methyltransferase

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ABSTRACT

***Hha*I DNA methyltransferase belongs to the C5-cytosine methyltransferase family, which is characterized by the presence of a set of highly conserved amino acids and motifs present in an invariant order. *Hha*I DNA methyltransferase has been subjected to a lot of biochemical and crystallographic studies. A number of issues, especially the role of the conserved amino acids in the methyltransferase activity, have not been addressed. Using sequence comparison and structural data, a structure-guided mutagenesis approach was undertaken, to assess the role of conserved amino acids in catalysis. Site-directed mutagenesis was performed on amino acids involved in cofactor *S*-adenosyl-ʟ-methionine (AdoMet) binding (Phe18, Trp41, Asp60 and Leu100). Characterization of these mutants, by *in vitro/in vivo* restriction assays and DNA/AdoMet binding studies, indicated that most of the residues present in the AdoMet-binding pocket were not absolutely essential. This study implies plasticity in the recognition of cofactor by *Hha*I DNA methyltransferase.**

INTRODUCTION

*Hha*I DNA methyltransferase (EC 2.1.1.37) is a part of the restriction–modification system of *Haemophilus haemolyticus* (1). The gene for the *Hha*I methylase was cloned, sequenced and found to code for a 327 amino acid protein (2). The protein has been overexpressed and purified in *Escherichia coli* (3–5). *Hha*I DNA methyltransferase (M.*Hha*I) is a C5-cytosine methyltransferase that recognizes the sequence 5′-GCGC-3′ in a double-stranded context and methylates the internal cytosine at the C5 position. Sequence alignment with the members of the C5-methyltransferase family revealed the presence of 10 conserved motifs, each containing about 5–10 amino acids, present in an invariant order in nearly all methyltransferases (6–8) including *Hha*I methyltransferase.

Among the 10 conserved motifs present in M.*Hha*I, two have been assigned a functional role: motif I, the FxGxG motif, found in all *S*-adenosyl-ʟ-methionine (AdoMet)-dependent methyltransferases and implicated in cofactor AdoMet binding (9); and motif IV, the PC motif involved in catalysis, where the sulfhydryl group of the conserved cysteine forms the covalent link with the target cytosine (10–12). Apart from motif I, the AdoMet-binding pocket is composed of a number of other amino acids from motifs II–V. The key interactions between the cofactor and the protein are mediated through a set of conserved amino acid residues, as seen from the ternary complex (12,13). The adenine ring of the AdoMet molecule is surrounded by hydrophobic amino acids, Phe18 (motif I) and Leu100 (motif V) on one side and Trp41 on the other. The FxGxG motif forms a tight loop in the first turn of a β_1–α_a–β_2 structural unit, with the first glycine being important in allowing a tight loop to form. Phe18 in this motif interacts with the adenosyl moiety of AdoMet, with its aromatic ring perpendicular to the plane of the purine ring. The ribose hydroxyl groups of AdoMet are hydrogen bonded by the acidic side chain of an invariant Glu40 from motif II. Asp60 (motif III) forms hydrogen bonds with the N6-amino group of adenine (8,14,15). The adenine ring of the cofactor is in hydrophobic interaction with Trp41, which lies parallel to the cofactor ring (π–π interaction) on one side and Leu100 and Phe18 on the other (8,12). The entire region surrounding the cofactor (motifs I, II and III) has resemblance to the Rossmann fold of the dinucleotide-binding motif (Fig. 1) (8,14). The DNA-binding region of M.*Hha*I is present within the variable region between motifs VIII and IX, and lacks any of the classical DNA-binding motifs.

Various crystal structures of M.*Hha*I, in binary complex with AdoMet or the reaction end product, *S*-adenosyl-ʟ-homocysteine (AdoHcy) or in a ternary complex with cofactor and different DNA substrates, have been determined (12–16). Based on these structures and those available for other methyltransferases, it was seen that the methyltransferases are organized into a two-domain structure. The larger domain comprises nine of the 10 conserved motifs (in the case of M.*Hha*I) and has the cofactor-binding domain and the catalytic motif. The variable region, between motifs IX and X, serves as the target recognition domain and forms the bulk of the smaller domain (17). Analysis of the crystal structures also revealed that the cofactor could be found in two different orientations. Based on kinetic analysis it was proposed that the two orientations could be related to the catalytically inactive unprimed and catalytically active primed orientations, as seen in the binary M.*Hha*I–AdoMet complex and ternary M.*Hha*I–DNA–AdoHcy complex or the binary M.*Hha*I–AdoMet complex in the presence of non-specific DNA, respectively (12,14,18).

For the M.*Hha*I reaction mechanism, which involves the formation of covalent enzyme–DNA complex and a dihydrocytosine intermediate, to be operational, a major distortion in the DNA would have to be evoked. It was indeed seen from the

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ternary complex of *Hha*I–DNA–AdoHcy (12) that the target cytosine was completely flipped out of the DNA double helix and placed in the enzyme active site.

*Hha*I DNA methyltransferase has been one of the most extensively studied methyltransferases in terms of the numerous crystal structures available (12–16,19). However, adequate mutational data to biochemically validate the crystallographic data is lacking. To date, only a few residues have been subjected to mutagenesis, namely Cys81, which forms a part of the conserved PC motif (20), Glu237, which fills the void created in the DNA as a result of base flipping (21), and Thr250, which is involved in DNA binding (22). Therefore, a structure-guided mutational analysis study of some of the conserved residues in M.*Hha*I was initiated. Site-directed mutagenesis was performed on residues present in the AdoMet-binding region. These mutants were analyzed in terms of their methylation activity, kinetic parameters and stability, using filter binding assays, fluorescence quenching and temperature dependence studies.

MATERIALS AND METHODS

Bacterial strains and plasmids

*Hha*I DNA methyltransferase was expressed in *E.coli* K12 strain ER1727 [(*mcr*BC-*hsd*RMS-*mrr*)2::Tn*10*, *mcr*A1272::Tn*10*, F′*lac proAB lac*I^q (*lac*Z)-M15]. *Escherichia coli* strain ER1546 (McrBC⁺) was used to check for the phenotype of the M.*Hha*I mutants. The M.*Hha*I gene was cloned into an IPTG inducible vector, pUHE25, (4). For carrying out site-directed mutagenesis the M.*Hha*I gene was cloned into pGEM-3Zf(+) (as a *Sph*I–*Hin*dIII fragment from pUHE25) which contains the f1 origin of replication. Single-stranded DNA for site-directed mutagenesis was prepared from *E.coli* strain ER2380 (*dut⁻ ung⁻* strain).

Protein purification

ER1727 cells containing the plasmid pUHE25 were grown to an absorbance (600 nm) of 0.8–1.0 at 37°C and subsequently induced with 0.15 mM IPTG. The cells were allowed to grow for 2 h after induction. The cells were harvested and either stored at –20°C or used immediately.

The protocol followed for the purification of M.*Hha*I was a slight modification of the earlier protocol (5). Cells were sonicated and the M.*Hha*I protein extracted from the cell pellet using buffer containing high salt (400 mM NaCl). The protein was then dialyzed with buffer containing 100 mM NaCl. The dialyzed protein was purified on Q-Sepharose and SP-Sepharose ion exchange columns. The purity of the protein was checked by SDS–PAGE with Coomassie brilliant blue staining (23). Protein was estimated using Coomassie G-250 and bovine serum albumin as standard (24).

Methylation activity

Filter binding. Methyltransferase activity of the M.*Hha*I was analyzed *in vitro* by measuring the incorporation of ³H-labeled methyl groups from [³H]AdoMet into substrate DNA (25). The reactions were carried out in 20 µl of methylase buffer, 50 mM Tris–HCl pH 7.5, 10 mM EDTA and 5 mM β-mercaptoethanol. Typically the reactions were performed with 1 µg λ DNA, 1–10 nM purified wild-type or mutant M.*Hha*I and 10–400 nM [³H]AdoMet at 37°C for 30 min. Reactions were

stopped by snap chilling in liquid nitrogen and then spotted onto DE-81 (Whatmann) anion exchange filters. These filters were then washed three times with cold 0.2 M ammonium bicarbonate and once with 100% ethanol. The filters were dried and analyzed by liquid scintillation counting.

In vivo Mcr restriction assay. Methyltransferase activity was also determined by the *in vivo* restriction assay (21,26). Plasmids harboring the wild-type or mutant M.*Hha*I gene were transformed into a McrBC⁺ (ER1564) or McrBC[−] (ER1727) strain of *E.coli*. The number of transformants obtained on a McrBC⁺ background was compared with those obtained on a McrBC[−] background.

DNA methylated by wild-type M.*Hha*I would be a substrate for the McrBC gene product and would therefore get restricted. Hence, no colonies would be obtained on a McrBC⁺ background upon transformation with wild-type or an active mutant methyltransferase.

In vitro restriction assay. The sensitivity of the plasmid DNA containing the wild-type or mutant M.*Hha*I gene to restriction digestion with its cognate endonuclease *Hha*I restriction endonuclease (R.*Hha*I) was also used to qualitatively assay for methylase activity. Plasmid DNA was isolated using the alkaline lysis method and subjected to digestion with R.*Hha*I at 37°C for 2 h. Samples were electrophoresed on a 1% agarose gel in TAE buffer and the DNA visualized by ethidium bromide staining and UV illumination. Plasmids expressing wild-type or active mutant methyltransferases would be resistant to digestion with R.*Hha*I due to methylation of the recognition sites.

Site-directed mutagenesis

The following oligonucleotides (Bangalore Genei, India) were used for site-directed mutagenesis: W41I, 5′-TTCTAATGAA-**ATCGAT**AAATATG-3′; W41A, 5′-TTCTAATGAA<u>TACG</u>-ATAAATATG-3′; W41Y, 5′-TTCTAATGAA<u>GCG</u>GATAA-ATATG-3′; W41R, 5′-TTCTAATGAA<u>AGG</u>GATAAATATG-3′; D60P, 5′-CCTGAGGGC<u>CCC</u>ATTACCCA-3′; D60N, 5′-CC-TGAGGGC<u>AAC</u>ATTACCCA-3′; L100S, 5′-AGAGGTAC-**AAGCTT**TTTTGAT-3′; F18L, 5′-TTTATTGACCTT<u>TTA</u>G-CAGGATTAGGT-3′; F18H, 5′-TTTATTGACCTT<u>CAT</u>-GCAGGATTAGGT-3′; F18X (X = Cys, Tyr or Ser), 5′-TT-TATTGACCTT<u>T(AGC)T</u>GCAGGATTAGGT-3′. The underlined sequences represent the site of mutation and the sequence in bold is the restriction site created for that particular mutation.

Mutagenesis was performed according to Kunkel's protocol for mutagenesis (27). Single-stranded DNA having uracil incorporated into it was made from *E.coli* strain ER2380 (*dut⁻ ung⁻*). Briefly, ER2380 was transformed with the pGEM3Z-f(+) clone of M.*Hha*I. Transformants were grown to an absorbance (600 nm) of 0.5 and infected with M13 helper phage, M13K07, with a multiplicity of infection of 10. The infected cultures were allowed to grow for another 8–10 h. M13K07 phage, packaged with single-stranded pGEM DNA, was subjected to PEG precipitation. Single-stranded DNA was isolated by phenol/chloroform treatment, followed by alcohol precipitation.

Phosphorylated oligonucleotides (using T4 polynucleotide kinase) were annealed to the single-stranded DNA by heating in a water bath to 80°C and subsequently cooling the mixture

to room temperature. Following annealing of the oligonucleotide, extension and ligation was carried out at 16°C for 12–16 h, using Klenow fragment of DNA polymerase I and T4 DNA ligase. This mix was transformed into an *E.coli* strain (*dut⁺ ung⁺*). The colonies were screened for the presence of mutations by the creation or loss of the restriction site as a result of mutation and by sequencing the appropriate region.

Immunoprecipitation of M.*Hha*I

Anti-*Hha*I antibodies were raised in rabbit using denatured M.*Hha*I protein. Immunoprecipitation was carried out essentially as described earlier (21). *Escherichia coli* ER1727 transformed with overexpression clones of wild-type or mutant M.*Hha*I were grown to an absorbance (600 nm) of 0.8 and induced with 0.15 mM IPTG. After 2 h the cells were harvested and the cell pellet resuspended in high salt buffer and briefly sonicated. The lysed cells were centrifuged for 10 min at 10 000 r.p.m. The supernatant was then collected and incubated with anti-M.*Hha*I antibodies for 3 h at 4°C with constant mixing. Protein A beads were added to this mix and the incubation continued for another 3 h. The beads were then pelleted and the immunoprecipitated protein was washed three times with low salt buffer. The amount of immunoprecipitated protein was estimated by western blotting using known amounts of purified wild-type protein. Protein A beads with bound *Hha*I methyltransferase were stored at 4°C and used directly in methylation assays.

Electrophoretic mobility shift assays

The following oligonucleotides (NEB, USA) were synthesized and used as a substrate for the mobility shift assays: 5′-GACTGGTACAGTATCAG**GCGC**TGACCCACAACAT-CCG-3′; 5′-TCGGATGTTGTGGGTCA**GCGC**CTGATACT-GTACCAGT-3′. The *Hha*I recognition sequence is in bold.

These complementary oligonucleotides were annealed to form duplexes by heating to 80°C and gradual cooling to room temperature. Either one of the oligonucleotides was end-labeled using T4 polynucleotide kinase and [γ-³²P]ATP, prior to annealing. Gel mobility shift assays were carried out in binding buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 13% glycerol, 7 mM β-mercaptoethanol and 100 μM AdoHcy). The assay conditions were essentially similar to those described previously (28). In a 10 μl reaction mix, M.*Hha*I was incubated with ³²P-labeled oligonucleotide in binding buffer for 10–15 min at 20°C. The protein–DNA complexes were analyzed on a 6% native polyacrylamide gel, which was pre-run for 30 min. Electrophoresis was performed in 90 mM Tris–borate pH 8.3, 2 mM EDTA, at room temperature for 1.5–2 h. The gels were dried on Whatmann 3 paper and autoradiographed on X-ray film or quantitated using a PhosphorImager.

Photoaffinity labeling of M.*Hha*I with [methyl-³H]AdoMet

Crosslinking of AdoMet to M.*Hha*I was done as described earlier (29). Wild-type or mutant M.*Hha*I (5 μM) was incubated with [methyl-³H]AdoMet (7.3 μM) in 10 μl of buffer (10 mM potassium phosphate, 5 mM EDTA, 10% glycerol and 0.1% 2-mercaptoethanol) for 10 min in a microtiter plate. A hand-held UV lamp was directly placed on the microtiter plate and the samples irradiated with UV for 60 min at 4°C. The samples were boiled in SDS loading buffer. The protein–AdoMet complex was analyzed by 12% SDS–PAGE, followed

by fixing in methanol:acetic acid:water (2.5:1:6.5) for 1 h. The fixed gel was subjected to fluorography (Amplify) for 30 min, dried and exposed to Kodak Hyperfilm at –70°C for 5 days.

Fluorescence titrations

The fluorescence emission spectra and the fluorescence intensities from titrations of M.*Hha*I were measured at 25°C on a FluoroMax3 spectrofluorimeter. The emission spectra were recorded over a wavelength of 300–400 nm with an excitation wavelength of 290 nm. Slit widths of 10 nm for excitation and 10 nm for emission were used. Titration of M.*Hha*I with AdoMet or AdoHcy was performed in low salt buffer. M.*Hha*I was allowed to equilibrate (5 min) in buffer before the beginning of the titration. Small aliquots of cofactor (final concentration 0.1–90 μM) were added to *Hha*I methyltransferase (1 μM) and incubated for 2 min before the spectra were recorded. The binding of AdoMet or AdoHcy to M.*Hha*I resulted in quenching of the Trp41 fluorescence, a unique amino acid present only at the AdoMet-binding site. Each spectrum recorded was an average of three scans. The fluorescence intensities were plotted against the total cofactor concentration and the data analyzed according to the Stern–Volmer and modified Stern–Volmer equations (30).

The Stern–Volmer relationship is represented by

$$F_o/F = 1 + K_{sv}[Q]$$

where F_o and F are the fluorescent intensities in the absence and presence of cofactor, respectively, K_{sv} is the collision Stern–Volmer constant and Q is the quencher (cofactor) concentration. In the case where there is a heterogeneous population of fluorophores, the modified Stern–Volmer relationship is used,

$$F_o/(F_o - F) = 1/\{[Q] f_a K_Q\} + 1/f_a$$

where f_a is the fractional number of fluorophores accessible to the quencher and K_Q is the quenching constant. The dissociation constants were calculated graphically using the modified Stern–Volmer plot (a plot of $F_o/(F_o - F)$ versus $1[Q]$) where $K_Q = 1/K_d$ (31,32).

Isothermal calorimetry (ITC) measurements

Titration calorimetric measurements were performed with a Microcal Omega titration calorimeter as described earlier (33). Briefly, small aliquots of AdoMet solutions at 15–40 times the M.*Hha*I concentration were added to a solution of M.*Hha*I (0.02–0.04 mM). The titrations were done under conditions that allowed for the precise estimate of the stoichiometry (*n*), binding constant (K_b) and enthalpy (ΔH°_b) simultaneously in a single ITC experiment. This was achieved by carrying out the titration under optimal '*c*' value (the *c* value is an arbitrary number defined as $c = K_b[\text{*Hha*I}]$ and for an optimal titration $100 < c < 1000$). Similar experiments were done with both the wild-type and mutant M.*Hha*I (W41I and W41Y).

The thermodynamic parameters were calculated using the following equations:

$$Q_t = n[\text{M.*Hha*I}]_t \ \Delta H^{\circ}_b V\{1 + [L]_t/n[\text{M.*Hha*I}]_t + 1/nK_b [\text{M.*Hha*I}]_t - [(1 + [L]_t/n[\text{M.*Hha*I}]_t + 1/nK_b[\text{M.*Hha*I}]_t)^2 - 4[L]_t/n [\text{M.*Hha*I}]_t]^{1/2}\}/2 \qquad \mathbf{1}$$

$$\Delta G^{\circ}_b = \Delta H^{\circ}_b - T\Delta S^{\circ}_b \qquad \mathbf{2}$$

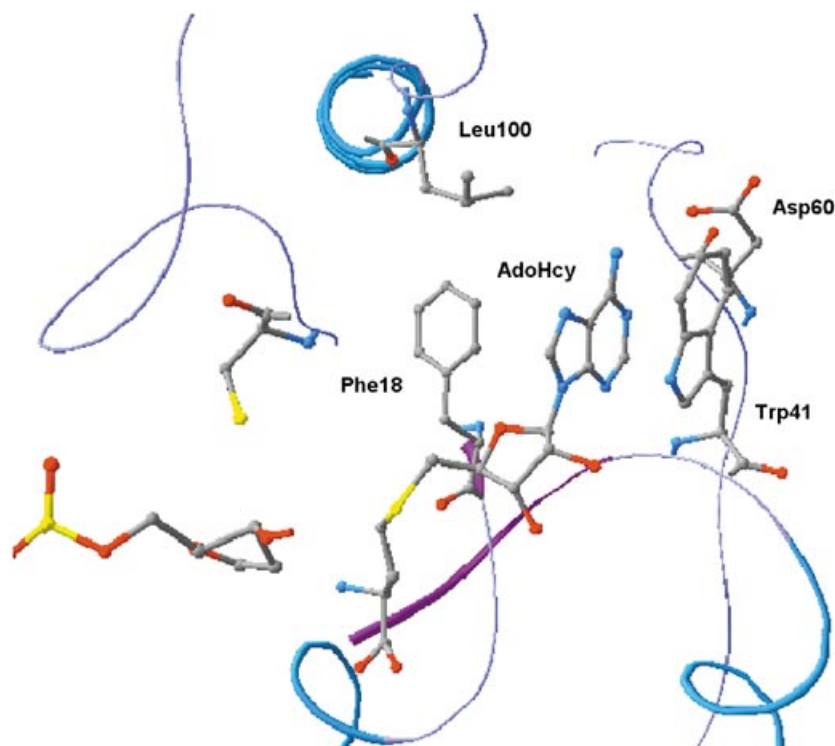$$\Delta G^{\circ}_b = -nRT \ln K_b \qquad \mathbf{3}$$

Figure 1. AdoMet binding pocket of *Hha*I DNA methyltransferase. The amino acid residues labeled have been subjected to mutagenesis.

where Q_t is the heat content of a solution, n is the stoichiometry, K_b is an intrinsic binding constant, ΔH°_b is an intrinsic heat of binding, [M.*Hha*I]$_t$ is the total M.*Hha*I concentration and V is the cell volume.

Specificity of methylation

The specificity of the mutant methyltransferase was determined by measuring the incorporation of [³H] label from AdoMet into premethylated DNA (by wild-type M.*Hha*I). DNA (λ) was methylated using excess cold AdoMet and wild-type enzyme. The methylation status of the DNA was examined by performing a restriction digestion of the DNA with R.*Hha*I. Methylation reactions were carried out as described above using the mutant methyltransferases (100 nM) and premethylated DNA or unmethylated DNA. The incorporation of radioactivity into premethylated DNA would suggest a change in specificity.

Stability of mutant methyltransferases

Stability of the wild-type and mutant methyltransferases was monitored by incubating the enzymes at 40°C in low salt buffer. Incubation was carried out at a protein concentration of 1 μM. At various time points, aliquots (2 μl) were withdrawn and assayed for methyltransferase activity at 37°C, using the DE81 filter binding assay as described earlier. The percent activity remaining was calculated and plotted against the incubation time. The stability of the mutant methyltransferases was also monitored by their rate of inactivation in the presence of urea. Wild-type or mutant M.*Hha*I (100 nM) was incubated with various concentrations of urea (0.1–4 M) in low salt buffer and methylation assays carried out at various concentrations of urea. The DE81 filter binding assay, described earlier,

was used to measure the activity of these enzymes. The experiments were performed three times in duplicate and the errors were within 5%.

RESULTS AND DISCUSSION

All C5-methyltransferases share a similar structural organization and contain motifs that are absolutely conserved throughout the family. It was, therefore, of interest to know whether the conserved residues are essential for methylase activity and, if so, to assign a possible role for these amino acids in catalysis. Mutations were made in the AdoMet-binding pocket of M.*Hha*I. The AdoMet-binding pocket comprises residues from the highly conserved motif I (FxGxG) and from motifs II–V (8). Residues involved in cofactor binding, Phe18, Trp41, Asp60 and Leu100, were subjected to mutagenesis (Fig. 1). Phe18 was changed to Ser, Cys, Tyr, Trp and His, Trp41 changed to Ala, Arg, Ile and Tyr, Asp60 changed to Pro and Asn, and Leu100 changed to Ser. The DNA-binding region of M.*Hha*I is present within the variable region between motifs VIII and IX, and lacks any of the classical DNA-binding motifs.

Activity of mutant methyltransferases

All the mutants were screened either by the loss or creation of a restriction site as a result of mutation. The mutations were confirmed by sequencing the appropriate regions of the M.*Hha*I gene.

The activity of the mutant methyltransferases was determined by both *in vivo* and *in vitro* restriction assays. Plasmid DNA encoding the wild-type or mutant methylase was simultaneously transformed into strains ER1727 (*mcrBC*⁻) and ER1564

Table 1. *In vivo* wild-type and mutant *Hha*I DNA methyltransferase activities

M.*Hha*I	Colonies (*mcr⁻/mcr⁺*)
Wild-type	102/0
F18S	58/0
F18C	72/88
F18Y	66/0
F18W	81/0
F18H	110/0
L100S	84/0
D60P	109/89
D60N	96/0
W41A	75/0
W41I	79/0
W41Y	72/0

Plasmids containing wild-type and mutant M.*Hha*I genes were transformed into an *E.coli* strain that expresses the *mcr*BC restriction system or a strain mutant for the *mcr*BC system, (ER1564 and ER1727, respectively). The number of colonies obtained on both the strains was compared. The numbers denoted are the average of three different experiments.

Table 2. *In vivo* methylation of *Hha*I recognition sequence by wild-type and mutant *Hha*I DNA methyltransferases

Enzyme	Sensitivity to R.*Hha*I[a]
Wild-type	–
W41A	–
W41R	–
W41I	–
W41Y	–
F18C	–
F18H	+/–
F18L	+/–
F18S	+/–
F18W	+/–
F18Y	+/–
L100S	–
D60P	+
D60N	–

Plasmid DNA containing the wild-type or mutant M.*Hha*I methyltransferase was subjected to digestion by R.*Hha*I. Plasmids containing an active methyltransferase would be resistant to digestion by R.*Hha*I whereas plasmids harboring an inactive methyltransferase would be cleaved by R.*Hha*I.
[a] –, resistance to cleavage by R.*Hha*I; +, complete digestion by R.*Hha*I; +/–, partial digestion by R.*Hha*I.

(*mcrBC⁺*) and the number of colonies obtained on each strain compared. The *E.coli* McrBC system encodes a methylation-sensitive restriction endonuclease, whose recognition sequence overlaps with the *Hha*I recognition sequence. Hence, plasmids carrying an active methylase would be restricted in *E.coli* strain ER1564 and not in strain ER1727. As can be seen from Table 1, all mutations at the Trp41 residue were active, and therefore no colonies were obtained on the McrBC⁺ strain. Mutations at the Phe18 residue also did not affect the activity of the methyltransferase, except for the Phe18→Cys mutant, which showed an inactive phenotype. Leu100→Ser and Asp60→Asn also did not have any affect on activity. However, mutation Asp60→Pro resulted in an inactive phenotype.

These results were further confirmed by the *in vitro* restriction assay. Plasmid DNA, from cells containing wild-type or mutant methylase, was isolated and its sensitivity to digestion by R.*Hha*I monitored. Plasmids harboring an active methylase would result in methylation of all *Hha*I sites *in vivo* and hence would be resistant to subsequent *in vitro* cleavage by R.*Hha*I. The results of the *in vitro* assay (Table 2) corroborated the *in vivo* McrBC restriction assay, except for the Phe18→Cys mutant, which was completely protected from endonuclease digestion. All other Phe18 mutants seemed to be partially protected. This suggested that all Phe18 mutants except Phe18→Cys were partially active. Plasmid DNA from cells expressing the Trp41 mutants, Asp60→Asn and Leu100→Ser mutants were protected from R.*Hha*I digestion and therefore are active methyltransferases, whereas the Asp60→Pro mutant was completely digested by R.*Hha*I, suggesting it to be an inactive methylase (Table 2).

Biochemical analysis

In order to assess the methylation activity of the mutants in a more quantitative manner, filter binding assays were performed to monitor incorporation of the [³H]methyl group into DNA. Either the purified or immunoprecipitated M.*Hha*I protein was used for the assays. Wild-type, Trp41 and Phe18 mutants were purified to homogeneity and subjected to biochemical analysis. The proteins were purified using a protocol similar to that used for the wild-type protein (5) (Fig. 2). The purified proteins were analyzed for methylation activity and AdoMet- and DNA-binding functions. The Asp60 and Leu100 mutants could not be purified due to either poor induction or insolubility of these mutants during the high salt extraction step of purification.

DNA binding and kinetic properties

To determine whether the Trp41 mutations had any effect on the DNA-binding properties, gel shift assays were performed using a 37mer oligonucleotide containing a single M.*Hha*I recognition site. End-labeled oligonucleotide was titrated with increasing concentrations of *Hha*I methyltransferase. The complex formed was resolved on native polyacrylamide gels and quantitated using a PhosphorImager. It was seen that the Trp41 mutants did not have any altered DNA-binding capabilities (Fig. 3).

As structural data implied an important role for Trp41 in cofactor binding, we investigated the effect of mutation at Trp41 on the kinetic properties of M.*Hha*I. Steady-state kinetic experiments were performed to obtain $K_{m(AdoMet)}$ and k_{cat} values using λ DNA and [methyl-³H]AdoMet as substrates (Table 3). Methyltransferase activity of Trp41 mutants (Ile, Tyr and Arg) did not vary appreciably from that of the wild-type protein. These mutations did not have a drastic effect on the $K_{m(AdoMet)}$ value (a maximum of 2-fold difference was seen with
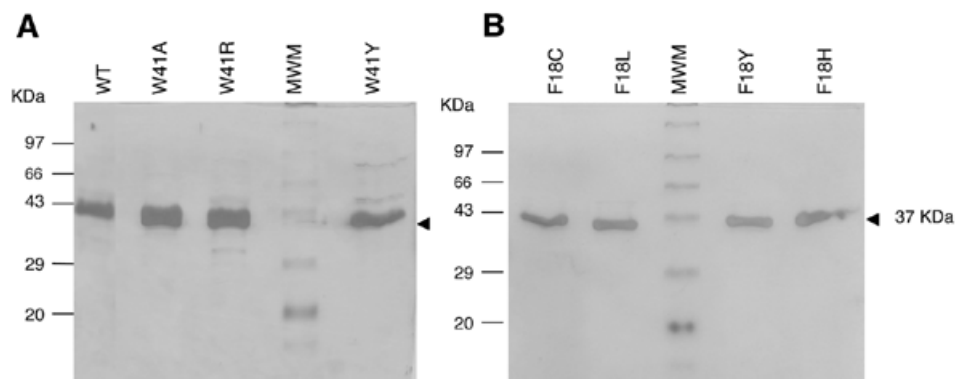
Figure 2. SDS–PAGE gel of purified wild-type and mutant *Hha*I DNA methyltransferases. Silver staining of 8−10 µg purified proteins run on 12% SDS−PAGE. (**A**) Trp41 mutants, (**B**) Phe18 mutants. MWM stands for standard molecular weight markers.
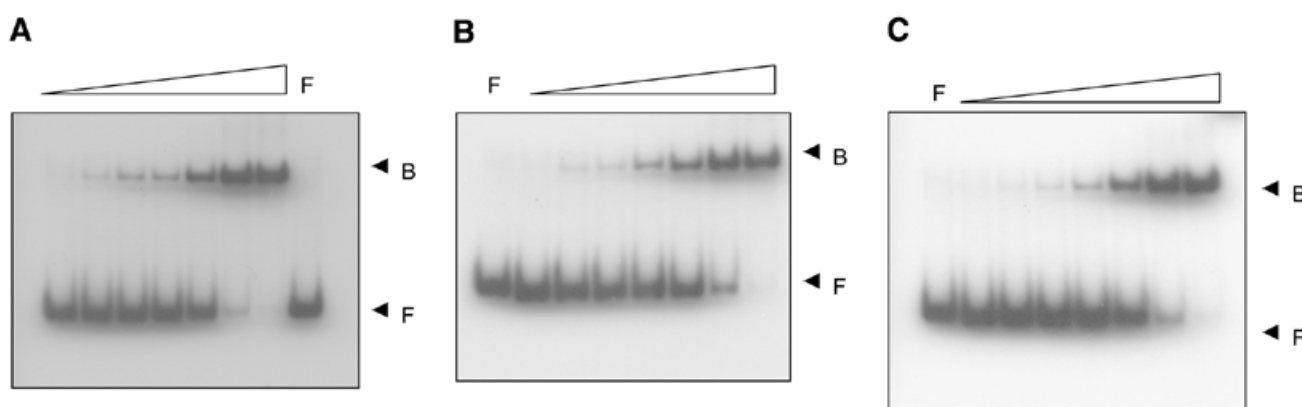

Figure 3. Binding of wild-type and mutant *Hha*I DNA methyltransferases to DNA. Electrophoretic mobility shift assays were performed using 5′-end-labeled duplex, containing a single recognition site for M.*Hha*I. Increasing concentrations of protein (1–250 nM) were added to a fixed amount of oligonucleotide (10 nM). The bound and the free oligonucleotides were separated by 6% native PAGE, in 0.5× TBE. The analysis was done for wild-type and Trp41 mutants. (**A**) Wild-type M.*Hha*I; (**B**) W41Y M.*Hha*I; (**C**) W41I M.*Hha*I. The arrows indicate protein−DNA complex (B) and free DNA (F).

Table 3. Kinetic parameters of wild-type and Trp41 mutants of *Hha*I DNA methyltransferase[a]

M.*Hha*I	$K_{m(AdoMet)}$ (nM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\times 10^{-3}$) (nM^{-1} min^{-1})
Wild-type	68.3	0.56	8.2
W41I	60.4	0.15	2.5
W41Y	70.9	0.57	8.0
W41R	25.0	0.24	9.5

[a]The average value of two independent experiments is reported.

Trp41Arg). The K_m values reflect the ability of AdoMet to interact with M.*Hha*I, whereas k_{cat} values are a reflection of the catalysis step. Substitution of Trp41 with Tyr did not change the enzyme properties significantly, as it is a conserved change, which can still maintain the π–π interaction with the adenine ring of AdoMet. Substitution with Ile however had an effect only on k_{cat} and not on K_m, i.e. only the catalysis step was affected by this mutation and there was no effect on cofactor binding. Ile could be maintaining the hydrophobic interactions necessary for cofactor binding, thus having no effect on K_m. The Arg mutation resulted in lowered K_m and k_{cat} values

(Table 3), suggesting that the charged residue could be interfering with both AdoMet binding and catalysis.

Both kinetic and gel shift assays indicate that the Trp residue at position 41 is dispensable as far as AdoMet binding and catalysis are concerned. This is not surprising as this Trp residue is not an absolutely conserved amino acid among the C5-methyltransferase family and other residues have been found to occupy a similar position (Met, Ile, Lys, Tyr, Asn, Phe, Leu and Gly) (12,15). The fact that this residue in not conserved undermines the importance of the hydrophobic interactions between Trp41 and the adenosyl moiety of AdoMet.

Methyltransferase activity was also measured for the Phe18 and Asp60 mutants. Since purification of some of these mutants proved to be difficult, immunoprecipitated protein was used for activity measurements (21). The activity of the immunoprecipitated protein (wild-type) was comparable with that of the normal soluble protein, indicating that the antibodies did not have an inhibitory effect on the methyltransferase activity. A comparison of the activities is shown in Figure 4. As can be seen, Phe18 mutants do retain some methyltransferase activity (5–20%). While the Asp60→Pro mutation led to a complete loss of activity (Fig. 4A), all Trp41 mutants retained wild-type-like activity (Fig. 4B). Subsequently,
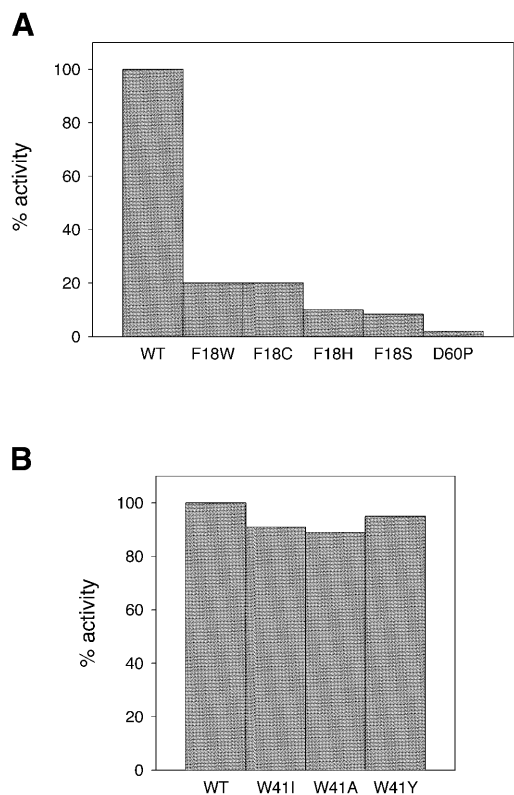
A

B

Figure 4. Activity of immunoprecipitated *Hha*I methyltransferases. Wild-type or mutant methyltransferases were precipitated from crude extract using anti-*Hha*I antibodies and protein A–Sepharose beads. The immunoprecipitated proteins were assayed for methyltransferase activity using the DE81 filter binding assay, as described in Materials and Methods. The figure shows activity for the mutant methyltransferases: (**A**) F18W, F18C, F18H, F18S and D60P; (**B**) W41I, W41A and W41Y. The activity shown is percentages normalized against wild-type M.*Hha*I. WT stands for wild-type.

we were able to purify some of the Phe18 mutants (Cys, Leu, His and Tyr). The purified proteins were used in later experiments.

AdoMet binding

Site-directed mutagenesis was carried out on residues involved in AdoMet binding. Most of the mutants do not result in the loss of methyltransferase activity (except Asp60→Pro), as seen by *in vivo* and *in vitro* restriction assay (Tables 1 and 2). As these mutations were in the AdoMet-binding pocket, they would be expected to have an effect, at least in their capacity to bind AdoMet and AdoHcy. The ability of these mutants to bind AdoMet was assessed by fluorescence quenching and isothermal titration calorimetry.

Fluorescence quenching. The presence of a Trp residue in the AdoMet-binding pocket of *Hha*I has been used as a handle to study cofactor binding. As can be seen, there was significant quenching of the fluorescence intensity of M.*Hha*I upon addition of AdoMet (Fig. 5A). This allowed for the quantitative measurement of the interaction of cofactor with *Hha*I methyltransferase. Wild-type or mutant M.*Hha*I (1 µM) was titrated with increasing concentrations of AdoMet (0.1–90 µM) and the fluorescence emission spectra were recorded between 300 and 400 nm. The fluorescence intensity at 354 nm was monitored and plotted against AdoMet concentration (Fig. 5A). The
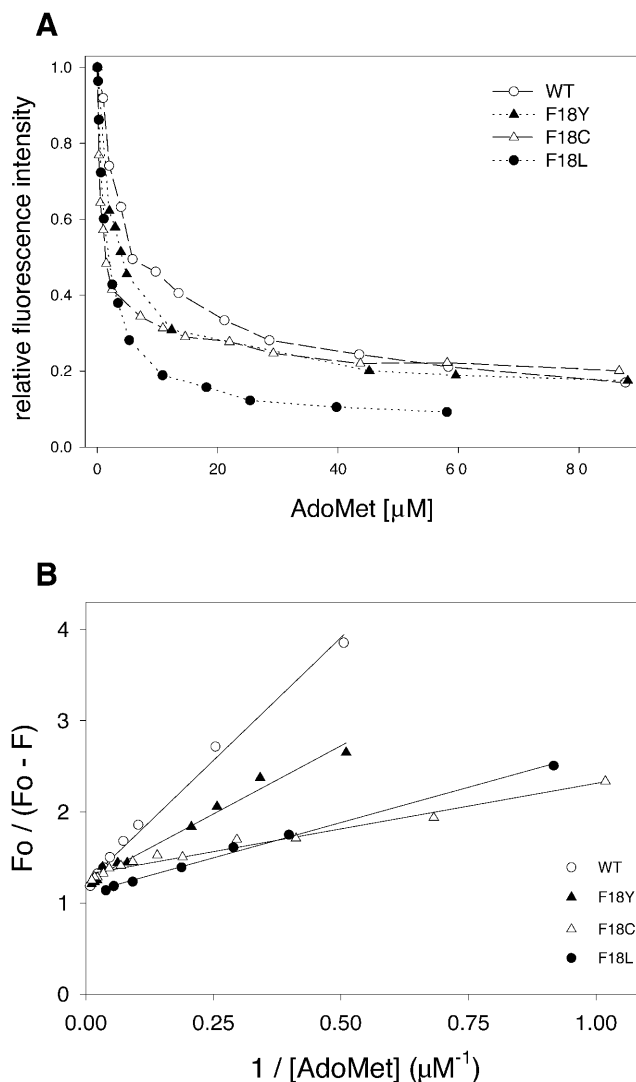
A

B

Figure 5. Tryptophan fluorescence analysis of the interaction of *Hha*I DNA methyltransferase with AdoMet. (**A**) Titration of 1 µM M.*Hha*I with AdoMet (0.1–90 µM). The relative fluorescence intensity (at 354 nm) (F/F_o) is plotted against AdoMet concentration for various mutant methyltransferases. (**B**) Fluorescence quenching data were analysed using the modified Stern–Volmer equation (see Materials and Methods) to obtain dissociation constants (K_d) for AdoMet.

data were analyzed using the modified Stern–Volmer plot (see Materials and Methods) (Fig. 5B). The dissociation constant (K_d) estimated for the wild-type *Hha*I was found to agree with the previously determined value using calorimetric (33) and fluorescence quenching studies (34). Similarly, the dissociation constants were estimated for the Phe18 mutants (Table 4). The affinity of these mutants for AdoMet increased 2- to 6-fold, the maximum being for the Phe18→Cys mutant.

ITC measurements. ITC measurements were carried out to characterize the thermodynamics of the interaction of cofactor (AdoMet or AdoHcy) with M.*Hha*I protein. ITC analysis done with wild-type M.*Hha*I indicated a dual mode of interaction with the cofactors (33). The primary high affinity mode could easily be saturated, while the secondary low affinity mode was

Table 4. Dissociation constants for wild-type and mutant *Hha*I DNA methyltransferases as determined by fluorescence quenching and ITC studies

Enzyme	Dissociation constant, K_d (µM)	
	Fluorescence[a]	ITC
Wild-type	4.5	6.34
F18C	0.76	ND
F18L	1.28	ND
F18Y	2.45	ND
W41I	ND	12.8
W41Y	ND	12.1

[a]Fluorescence measurements were not performed with the Trp41 mutants as M.*Hha*I has a single Trp residue, the loss of which results in decreased fluorescence.
ND, not determined.

not saturated within the concentration range of AdoMet used for titration. Hence, the thermodynamic parameters were estimated by analyzing only the primary mode (33). The dissociation constant obtained from such an analysis was similar to that obtained by fluorescence quenching studies (34). Two Trp41 mutants, Trp41→Ile and Trp41→Tyr, were also subjected to calorimetric analysis. Both these mutants displayed a dual mode interaction, as seen for the wild-type enzyme. A representative binding isotherm, for the primary mode, for the wild-type and Trp41→Ile mutant is shown in Figure 6. A similar binding isotherm was obtained for Trp41→Tyr. The binding affinities estimated from the isothermal titration calorimetry experiments for both these mutants were 2-fold lower compared with the wild-type protein (Table 4).

It can, therefore, be concluded from the AdoMet binding studies that mutations at the Trp41 residue do not significantly affect the M.*Hha*I–cofactor interaction. However, mutations at Phe18 resulted in an enhanced binding of AdoMet, as seen from the fluorescence quenching data (Table 4).

Methyltransferase specificity and stability

In order to check whether the mutant methyltransferases have any altered specificities, methylation reactions were carried out using premethylated DNA. Incorporation of radioactivity into DNA premethylated by wild-type *Hha*I would indicate a change in specificity. Typically, 100 nM enzyme was incubated with premethylated λ DNA in the presence of 200 nM [³H]AdoMet. The methylation reaction with unmethylated DNA was used as a control. As can be seen (Table 5), the wild-type M.*Hha*I was not capable of methylating premethylated DNA. Similarly, among all the mutant methylases analyzed, those which were partially active were incapable of further methylating the premethylated DNA, thus confirming that there was no change in specificity of the mutant methyltransferases.

Having seen that there was no altered specificity of these mutant methyltransferases, we next went ahead to see if the thermal stability of the mutants was affected. The stability of the mutant methyltransferases was assayed by incubating the enzymes at 40°C and assaying for methyltransferase activity at 37°C at the indicated time points. The fraction inactivation was
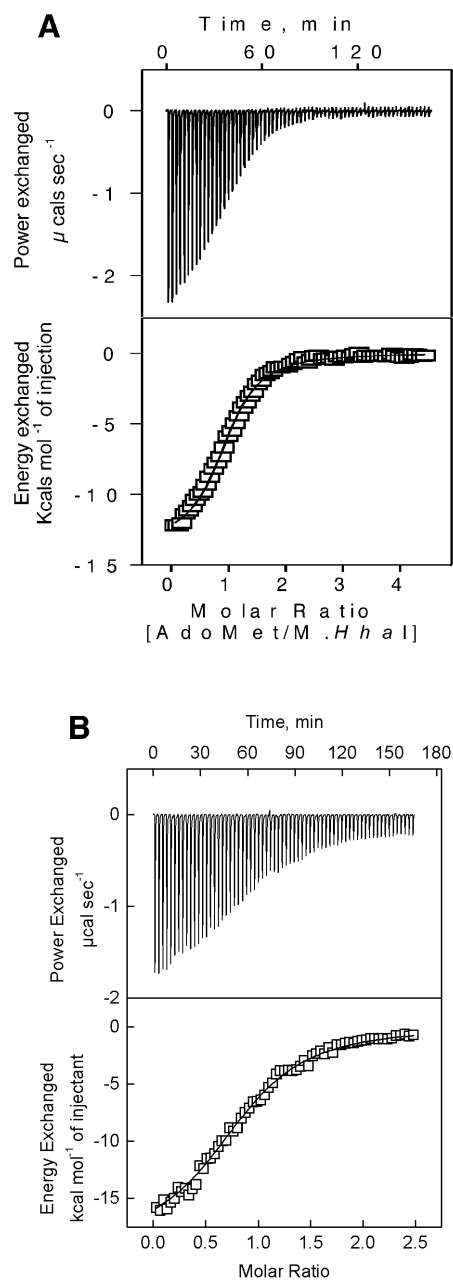

Figure 6. Isothermal titration calorimetric analysis of wild-type and Trp41 mutant of *Hha*I DNA methyltransferase. Titrations were performed as mentioned in Materials and Methods. The primary endothermic binding mode depicting the heat absorbed as a function of increasing AdoMet/M.*Hha*I ratio for (**A**) wild-type M.*Hha*I and (**B**) Trp41Ile mutant. The top panel represents the raw data obtained for each addition of AdoMet to M.*Hha*I. The bottom panel is the non-linear least squares fit of the incremental heat per mole of added ligand (AdoMet). Using these heats, the enthalpy, free energy and binding constants were derived.

plotted against the incubation time (Fig. 7). Results indicate that the Trp41 mutant (Ile, Ala and Tyr) methyltransferases had slightly increased stability as compared with the wild-type enzyme (Fig. 7A). In contrast, the Phe18 mutants were found to be unstable (except Phe18→Cys) compared with the wild-type protein (Fig. 7B). The Phe18→Tyr and Phe18→His mutants were very unstable and by 20 min lost activity

Table 5. Specificity of methyltransferases

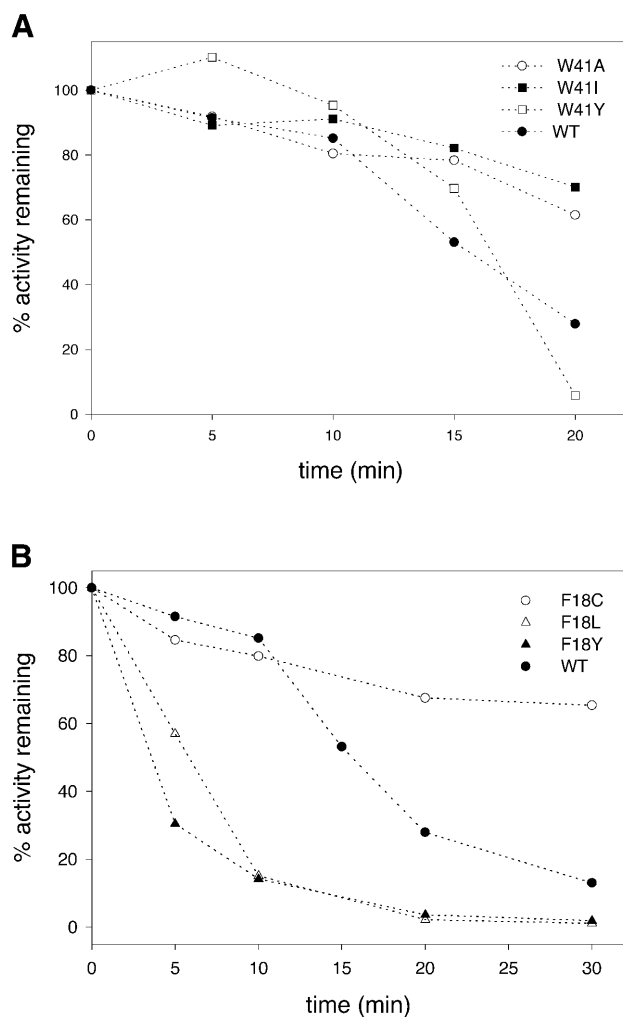| Enzyme	c.p.m. (DNA^{me})	c.p.m. (DNA)
Wild-type	105	14 048
W41A	182	1482
W41I	115	8131
W41Y	76	13 752
F18C	60	6055
F18H	61	169
F18L	113	2024
F18Y	110	1322

A

B

Figure 7. Kinetics of inactivation of wild-type and mutant *Hha*I DNA methyltransferases. Wild-type or mutant M.*Hha*I (1 µM) were incubated at 40°C in low salt buffer (see Materials and Methods). Aliquots (2 µl) were removed at various times and assayed for methylase activity, as described in Materials and Methods. A plot of activity versus incubation time is shown: (**A**) Trp41 mutant methyltransferases; (**B**) Phe18 mutant methyltransferases. The figure shows activity as percentages normalized against the wild-type M.*Hha*I. WT stands for wild-type.

completely. Stability of the mutant methyltransferases was also determined by their resistance to urea denaturation. Methylation activity of the mutants was measured in the presence of various
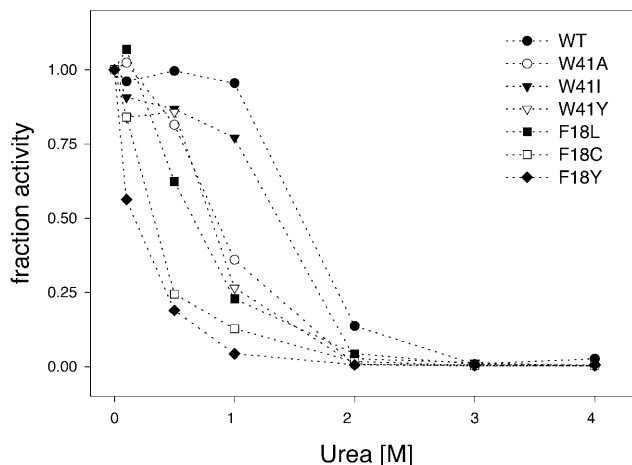
Figure 8. Denaturation of wild-type and mutant *Hha*I DNA methyltransferases in the presence of urea. M.*Hha*I (100 nM) was incubated with urea (0.1–4 M) for 5 min at 37°C and the activity of the protein measured in the presence of denaturant. Fractional activity plotted against urea concentration for Trp41 mutants (Ala, Ile and Tyr) and Phe18 mutants (Leu, Cys and Tyr) is shown.

concentrations of urea. As can be seen (Fig. 8), all the mutants (Trp41 and Phe18) were less stable compared with the wild-type protein. The effect was more prominent in the case of the Phe18 mutants. The Phe18→Cys and Phe18→Tyr mutants were the least stable and, at a concentration of 0.5 M urea, lost 75% of their activity.

Phe18 is a part of motif I, FxGxG, and, except in one or two cases, is absolutely conserved among all AdoMet-dependent methyltransferases. It is, therefore, proposed to play a role in cofactor binding (8,9). In the case of M.*Hha*I, the Phe18 ring lies perpendicular to the adenine ring of AdoMet, thus interacting with the cofactor through hydrophobic interactions. Results obtained in this study show that changes at Phe18 result in partial loss of activity. No apparent correlation could be drawn between the nature of the side chains and enzymatic activity. Substitutions with either a bulky or a smaller side chain could result in disruption of the interaction with AdoMet, thus affecting the methyltransferase activity. On the other hand, substitutions at Phe18 also seem to have an effect on the stability of the mutant methylase (Fig. 8). These results, therefore, suggest that the loss of stability could also be responsible for the reduced activity seen. However, it is not clear why the affinity for the cofactor AdoMet is increased. One possible reason for the reduced activity of the mutants is the inhibitory effect due to the increased affinity for cofactors. Although K_d values for AdoHcy are not available, they would be expected to follow a trend similar to the wild-type enzyme, i.e. the values would be higher for the wild-type compared with the mutants. This increased affinity for the reaction end product, AdoHcy, could affect the product release step during catalysis. Among the Phe18 mutants, Phe18→Cys showed anomalous behavior, as seen by the *in vitro* and *in vivo* restriction assays, and was found to be more stable to heat denaturation. At present we are unable to explain these observations.

In summary, this mutational study indicates that amino acid residues involved in the binding of the cofactor AdoMet are not absolutely essential and suggests flexibility in the AdoMet-binding pocket (Table 6). The hydrophobic pocket formed by

Table 6. Properties of wild-type and mutant M.*Hha*I

Enzyme	*In vitro* assay[a]	*In vivo* assay[a]	Methylase activity[b]	AdoMet binding[c]		Stability to heat/urea[d]
				Crosslinking	K_d[b]	
Wild–type	+	+	1	+	1	++/++
Trp41						
W41A	+	+	0.74	+/–	ND	++/+
W41I	+	+	0.58	+/–	2	++/+
W41R	+	+	0.52	+/–	ND	
W41Y	+	+	0.97	+/–	1.9	++/+
Phe18						
F18C	+	–	0.43	+/–	0.17	++/+
F18L	+/–	+	0.14	+	0.28	+/+
F18Y	+/–	+	0.73	+/–	0.54	+/+
F18H	+/–	+	0.01	+/–	ND	ND
F18S	+/–	+				
F18W	+/–	+				
Asp60						
D60P	–	–	These mutant methyltransferases could not be purified due to the insoluble nature of these proteins			
D60N	–	–				
Leu100						
L100S	+	+				

[a]+, –, +/–, active, inactive and partially active, respectively.
[b]Values relative to wild-type M.*Hha*I.
[c]AdoMet binding studied qualitatively by crosslinking (+/–, weak; +, strong) or quantitatively by ITC (Trp41 mutants) or fluorescence quenching (Phe18 mutants).
[d]++, stable; + unstable; ND, not determined.

Phe18, Leu100 and Trp41 may not be as important as expected from structural analysis and this could be a peculiarity of M.*Hha*I. It is possible that the size of the side chain plays a more important role than the specific nature of interactions with the cofactor. On the other hand, the stability of some of the Phe18 mutants was affected, suggesting an additional structural role for this amino acid apart from a functional one. The absence of absolute conservation at these (expected) important residues suggests to us that the enzyme may be using an alternative mode to interact with the cofactor. Our mutational analysis and those done earlier (21,22) suggest that M.*Hha*I is more tolerant to changes at the conserved residues or residues thought to have an important functional role.

ACKNOWLEDGEMENTS

REFERENCES

1. Roberts,R.J., Myers,P.A., Morrison,A. and Murray,K. (1976) A specific endonuclease from *Haemophilus haemolyticus*. *J. Mol. Biol.*, **103**, 199–208.
2. Caserta,M., Zacharias,W., Nwankwo,D., Wilson,G.G. and Wells,R.D. (1987) Cloning, sequencing, *in vivo* promoter mapping and expression in *Escherichia coli* of the gene for the *Hha*I methyltransferase. *J. Biol. Chem.*, **262**, 4770–4777.
3. Wu,J.C. and Santi,D.V. (1988) High level expression and purification of *Hha*I methyltransferase. *Nucleic Acids Res.*, **16**, 703–717.
4. Klimasauskas,S., Nelson,J.L. and Roberts,R.J. (1991) The sequence specificity domain of cytosine-C5 methylases. *Nucleic Acids Res.*, **19**, 6183–6190.
5. Kumar,S., Cheng,X., Pflugrath,J.W. and Roberts,R.J. (1992) Purification, crystallization and preliminary X-ray diffraction analysis of an M.*Hha*I–AdoMet complex. *Biochemistry*, **31**, 8648–8653.
6. Posfai,J., Bhagwat,A.S., Posfai,G. and Roberts,R.J. (1989) Predictive motifs derived from cytosine methyltransferases. *Nucleic Acids Res.*, **17**, 2421–2435.
7. Cheng,X., Kumar,S., Klimasauskas,S. and Roberts,R.J. (1993) Crystal structure of the *Hha*I DNA methyltransferase. *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 331–338.
8. Kumar,S., Cheng,X., Klimasauskas,S., Mi,S., Posfai,J., Roberts,R.J. and Wilson,G.G. (1994) The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.*, **22**, 1–10.
9. Ingrosso,D., Fowler,A.V., Bleibaum,J. and Clarke,S. (1989) Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. Common sequence motifs for protein, DNA, RNA and small molecule S-adenosylmethionine-dependent methyltransferases. *J. Biol. Chem.*, **264**, 20131–20139.
10. Wu,J.C. and Santi,D.V. (1987) Kinetic and catalytic mechanism of *Hha*I methyltransferase. *J. Biol. Chem.*, **262**, 4778–4786.

11. Chen,L., MacMillan,A.M., Chang,W., Ezaz-Nikpay,K., Lane,W.S. and Verdine,G.L. (1991) Direct identification of the active-site nucleophile in a DNA (cytosine-5)-methyltransferase. *Biochemistry*, **30**, 11018–11025.

12. Klimasauskas,S., Kumar,S., Roberts,R.J. and Cheng,X. (1994) *Hha*I methyltransferase flips its target base out of the DNA helix. *Cell*, **76**, 357–369.

13. O'Gara,M., Klimasauskas,S., Roberts,R.J. and Cheng,X. (1996) Enzymatic C5-cytosine methylation of DNA: mechanistic implications of new crystal structures for *Hha*I methyltransferase–DNA–AdoHcy complexes. *J. Mol. Biol.*, **261**, 634–645.

14. O'Gara,M., Zhang,X., Roberts,R.J. and Cheng,X. (1999) Structure of a binary complex of *Hha*I methyltransferase with S-adenosyl-L-methionine formed in the presence of a short non-specific DNA oligonucleotide. *J. Mol. Biol.*, **287**, 201–209.

15. Cheng,X., Kumar,S., Posfai,J., Pflugrath,J.W. and Roberts,R.J. (1993) Crystal structure of the *Hha*I DNA methyltransferase complexed with S-adenosyl-L-methionine. *Cell*, **74**, 299–307.

16. Kumar,S., Horton,J.R., Jones,G.D., Walker,R.T., Roberts,R.J. and Cheng,X. (1997) DNA containing 4′-thio-2′-deoxycytidine inhibits methylation by *Hha*I methyltransferase. *Nucleic Acids Res.*, **25**, 2773–2783.

17. Cheng,X. (1995) Structure and function of DNA methyltransferases. *Annu. Rev. Biophys. Biomol. Struct.*, **24**, 293–318.

18. Lindstrom,W.M.,Jr, Flynn,J. and Reich,N.O. (2000) Reconciling structure and function in *Hha*I DNA cytosine-C-5 methyltransferase. *J. Biol. Chem.*, **275**, 4912–4919.

19. O'Gara,M., Horton,J.R., Roberts,R.J. and Cheng,X. (1998) Structures of *Hha*I methyltransferase complexed with substrates containing mismatches at the target base. *Nature Struct. Biol.*, **5**, 872–877.

20. Mi,S. and Roberts,R.J. (1993) The DNA binding affinity of *Hha*I methylase is increased by a single amino acid substitution in the catalytic center. *Nucleic Acids Res.*, **21**, 2459–2464.

21. Mi,S., Alonso,D. and Roberts,R.J. (1995) Functional analysis of Gln-237 mutants of *Hha*I methyltransferase. *Nucleic Acids Res.*, **23**, 620–627.

22. Vilkaitis,G., Dong,A., Weinhold,E., Cheng,X. and Klimasauskas,S. (2000) Functional roles of the conserved threonine 250 in the target recognition domain of *Hha*I DNA methyltransferase. *J. Biol. Chem.*, **275**, 38722–38730.

23. Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

24. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

25. Mi,S. and Roberts,R.J. (1992) How M.*Msp*I and M.*Hpa*II decide which base to methylate. *Nucleic Acids Res.*, **20**, 4811–4816.

26. Raleigh,E.A., Trimarchi,R. and Revel,H. (1989) Genetic and physical mapping of the *mcr*A (rglA) and *mcr*B (rglB) loci of *Escherichia coli* K-12. *Genetics*, **122**, 279–296.

27. Kunkel,T.A. (1985) The mutational specificity of DNA polymerases-alpha and -gamma during *in vitro* DNA synthesis. *J. Biol. Chem.*, **260**, 12866–12874.

28. Dubey,A.K. and Roberts,R.J. (1992) Sequence-specific DNA binding by the *Msp*I DNA methyltransferase. *Nucleic Acids Res.*, **20**, 3167–3173.

29. Ahmad,I. and Rao,D.N. (1994) Photolabeling of the *Eco*P15 DNA methyltransferase with S-adenosyl-L-methionine. *Gene*, **142**, 67–71.

30. Lehrer,S.S. (1971) Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry*, **10**, 3254–3263.

31. Samworth,C.M., Degli Esposti,M. and Lenaz,G. (1988) Quenching of the intrinsic tryptophan fluorescence of mitochondrial ubiquinol–cytochrome-c reductase by the binding of ubiquinone. *Eur. J. Biochem.*, **171**, 81–86.

32. Lackowicz,J.R. (1983) *Principles of Fluorescence Spectroscopy*. Plenum Press, NY.

33. Swaminathan,C.P., Sankpal,U.T., Rao,D.N. and Surolia,A. (2002) Water-assisted dual mode cofactor recognition by *Hha*I DNA methyltransferase. *J. Biol. Chem.*, **277**, 4042–4049.

34. Vilkaitis,G., Merkiene,E., Serva,S., Weinhold,E. and Klimasauskas,S. (2001) The mechanism of DNA cytosine-5 methylation. Kinetic and mutational dissection of *Hha*I methyltransferase. *J. Biol. Chem.*, **276**, 20924–20934.