The DNA binding affinity of HhaI methylase is increased by a single amino acid substitution in the catalytic center

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Received January 19, 1993; Revised and Accepted April 6, 1993

ABSTRACT
The HhaI methyltransferase recognizes the sequence GCGC and transfers a methyl group to C5 of the first cytosine residue. All m5C-methyltransferases contain a highly conserved sequence motif called the P-C motif. The cysteine residue of this motif is involved in catalysis by forming a covalent bond with the 5-position of cytosine prior to methyl group transfer. For the EcoRI methyltransferase, it has been shown that substitution of this catalytic cysteine by glycine is cytotoxic to E.coli. We now show that this observation can be extended to the HhaI system and suggest that the cytotoxicity is due to abnormally tight DNA binding by the mutant methyltransferase, which probably interferes with replication or transcription.

INTRODUCTION
5-methylcytosine methyltransferases (m5C-methyltransferases) are present in organisms ranging from bacteria to mammals (1). These enzymes transfer a methyl group from S-adenosyl-methionine (SAM) to the C5 position of a cytosine residue in a specific double-stranded DNA sequence. The family of m5C-methyltransferases shares an overall common architecture: there are five highly conserved motifs about 10 to 20 amino acids long and five moderately conserved motifs (2). Motif I, located near the N-terminus, has a close relative in many other families of protein, DNA and RNA methyltransferases, and may be important for SAM interaction (3,4). Recognition of a specific DNA sequence by the methyltransferases is mediated by a ‘variable region’ that lies between conserved motifs VIII and IX (5,6). Motif IV, called the P-C motif, is part of the catalytic center of the enzyme. It has been proposed by Santi (7) that cytosine methyltransferases use a mechanism analogous to that employed by thymidylate synthase (8) in which an early reaction step involves formation of a transient covalent protein-DNA intermediate between a Cys residue in the enzyme and the C6 position of cytosine (7,9). The methyl group, donated by SAM, is transferred to the C5 position. Several studies have shown that cytosine methyltransferases interact with DNA containing 5-azacytidine (5-azaC) or 5-fluorocytosine, both potent inhibitors of m5C-methyltransferases, to form a covalent complex with the enzyme (10,11). Amino acid Cys-71, located in the P-C motif of the HaeIII methyltransferase, has been identified recently as the active catalytic site (10).

It was first observed for the SPR multi-specific methyltransferase that replacement of the cysteine residue in the P-C motif by serine destroyed catalytic activity (12). A later study of the EcoRI methyltransferase (M.EcoRI, recognition sequence CCWGG), showed that replacement of the conserved cysteine with serine, valine or tryptophan abolished catalysis (13). A glycine substitution both abolished catalysis and proved cytotoxic to E.coli (13). In this paper we show that HhaI methyltransferase (M.HhaI, recognition sequence GCGC) exhibits similar properties when the conserved Cys-81 in the P-C motif is altered. Mutants in which Cys-81 was replaced with Arg, His or Ser only lost methyltransferase activity. In contrast, the Gly-81 mutant lost activity and was cytotoxic. A biochemical basis for this cytotoxicity was sought. Here, we demonstrate that the Gly-81 substitution lowered the K_d of the enzyme for its DNA substrate about 3-fold and its k_cat about 50-fold. This enhanced affinity for target DNA probably interferes with cellular DNA replication or transcription causing cytotoxicity.

MATERIALS AND METHODS
Bacterial strains and plasmids
Escherichia coli K-12 strains: ER1727 [Δ(mcrB-mrr)102::Tn10, mcrA1272::Tn10, F' lac pro AB lacF'Q (lacZ) M15] was kindly provided by E. Raleigh. Plasmid pHSH01, which encodes the H0 methyltransferase (5), contains a mutant form (I2L) of M.HhaI under the control of the T7 promoter. The properties of this mutant are indistinguishable from wild-type (5). Plasmid pAIT contains M.SsrI and was kindly provided by W.Jack (New England Biolabs). Plasmid pGEM-3Zf(+) containing the fl origin, used for preparation of single stranded DNA for site directed mutagenesis, was purchased from Promega.

Enzymes and reagents
Restriction enzymes, T4 DNA ligase, Vent DNA polymerase and bacteriophage lambda phage DNA were from New England Biolabs and used according to the manufacturer’s specifications. Deoxyctydine-5'[^32P]-triphosphate and deoxyadenosine-
5'[^32P]-triphosphate (6000 Ci/mmmole) were from Amersham. DNA sequencing was performed using a kit from United States Biochemicals and site-directed mutagenesis was performed using a kit from Amersham. S-adenosyl-L[3H]methionine (15 Ci/mmmole) was from Amersham.

Site-directed mutagenesis

The following primers were synthesized by the Midland certified reagent company for use in mutagenesis.

A: TGTGCGAGGTTTCGCCACCAAGCCTTT
B: GCAGGAGTTCCGCCGACAGCCTTT

The underlined codon CAC in primer A encodes a histidine. Oligonucleotide B is degenerate at the position marked V (A/G/C) to generate codons encoding either serine (AGC), glycine (GGC), or arginine (CGC). Plasmid pGEM3Zf(+)+Hhal was constructed by subcloning the Spfl-HindIII fragment containing the whole reading frame of the M.Hhal gene from plasmid pHSHO-1 into pGEM3Zf(+). Plasmids pGEM3Zf(+)+M.Hhal-His81, -Ser81, -Gly81 and -Arg81 contain mutations of Cys81 made by oligonucleotide directed mutagenesis (14) using oligonucleotides A or B. Mutations were confirmed by dye-deoxy chain termination sequencing. Plasmid pUHE25 is an expression vector carrying the T7 early promoter/lac operator sequence (15) and was a gift of U. Deuschle. Plasmids pUHE25Gly-81, pUHE25Arg-81, pUHE25Ser-81 and pUHE25His-81 were constructed by ligating the Spfl-HindIII fragment from pGEM3Zf(+)+M.Hhal-Gly-81, Ser-81, Arg-81 and His-81, respectively, into identical sites within plasmid pUHE25.

Purification of M.Hhal and mutants

The Arg-81, Ser-81 and His-81 mutants of M.Hhal were purified as described by Kumar et al. (16) except that no SAM was added. The following method was used to purify the Gly-81 mutant. Plasmid pUHE25Gly-81 and plasmid pAIT2, expressing M.Sssl, were co-transformed into ER1727 cells and selected using the plasmid pUHE25.

Mobility shift assays

The following oligonucleotides for use in gel retardation assays were synthesized at New England Biolabs or in the Cold Spring Harbor Oligonucleotide facility.

1. 5'- GAC TGG TAC AGT ATC AGG CCC TGA ACC ACA ACA TCC G-3'
2. 5'- GAC TGG TAC AGT ATC AGG CCC TGA ACC ACA ACA TCC G-3'
3. 5'- TCG GAT GTT GGT GAT GCC GGG TCA CCT TGA GG-3'
4. 5'- TCG GAT GTT GGT GAT GCC GGG TCA CCT TGA GG-3'
5. 5'- TGC AGT CCC GAT GCC GGG TCA CCT TGA GG-3'
6. 5'- TTC AGC GCT ACC CCC GAC TGG TAC ACC GT-3'

The underlined regions of the oligonucleotides represent the methyltransferase recognition sites. Oligos 1 and 3 are complementary 37-mers used for the specific DNA binding assay. Oligos 2 and 4 are complementary 29-mers used to detect non-specific binding. Oligos 5 and 6 are complementary 37-mers that in conjunction with oligos 1 and 3 can form either hemi-methylated or fully methylated duplexes. To form duplexes, appropriate complementary oligonucleotides were annealed by heating at 80°C and gradually cooled to room temperature. Purification of oligonucleotides, labeling procedures and DNA-binding assays were performed as described previously (19) except that the DNA was labeled with [α-32P]dATP and [α-32P]dCTP with a specific activity of 6000Ci/mmole.

Complex dissociation

Complexes between the purified methyltransferase and the labeled, unmethylated duplex 37-mer (oligos #1 and 3) were formed as described previously (19). Briefly, the binding reaction was carried out in 10 μl binding buffer (50 mM Tris.HCl (pH 8.0), 10 mM Na2EDTA, 7 mM β-mercaptoethanol, 100 μM SAH, and 13% glycerol. Unless indicated otherwise, 27 nM methyltransferase and 81 nM radiolabelled DNA probe were used. The reaction was incubated at 22°C for 30 mins. and the unlabeled DNA (8.1 μM) was added at timed intervals as indicated in Figure 4. The complexes were analyzed by electrophoresis on native 10% polyacrylamide gels run in TBE (89 mM Tris.borate (pH 8.0), 2 mM Na2EDTA). The gel was prerun at 100 volts for 60 mins. Following electrophoresis the gel was dried and analyzed using a Phosphor-Imager (Molecular Dynamics) and also by autoradiography.

RESULTS

Mutant methyltransferases and cell growth

Site directed mutagenesis was used to replace the conserved cysteine at position 81 in the P-C motif of M.Hhal. The mutant methyltransferases were expressed from the early T7 promoter under the control of the lac operator (15). Expression of the methyltransferases was induced by IPTG, and their effects on cell growth were monitored. Cells were removed at various times after induction and replated, without induction, to test the number of viable cells remaining. The results are shown in Figure 1. Cells

Assays for methylation activity

a. Sensitivity to restriction endonucleases. Plasmid DNAs, carrying wild-type or mutant methyltransferases were isolated using the alkaline lysis method (17), further purified on Qiagen columns and then digested with an excess of the Hhal restriction endonuclease (R.Hhal). Typically 0.3 μg plasmid DNA was digested with 20 units R.Hhal for 2 hrs at 37°C. The digestion products were analyzed by 1% agarose gel electrophoresis in the presence of ethidium bromide.

b. In vitro methylation activity. Bacteriophage lambda DNA, digested with R.HindIII was incubated in the presence of [3H]-SAM with various purified methyltransferase proteins. Briefly the reaction was performed in 20 μl methylation buffer containing 50 mM Tris.HCl (pH 7.5), 10 mM Na2EDTA, 5 mM b-mercaptoethanol, 65 μM [3H]-SAM typically using 54 nM methyltransferase and 1 μg DNA. Samples were incubated at 37°C for 60 mins. The incubation mixture was then treated with proteinase K at 65°C for 20 mins. and analyzed by a DE81 filter binding assay (18).
R.Hhal by in vitro. Figure 2 shows protecting it from digestion in vivo, thereby active sensitivity to digestion by in vitro. R.Hhal was determined the wild-type or mutant methyltransferase were isolated and their activity to digestion by in vitro. First, plasmid DNAs from cells expressing mutant methyltransferases were isolated and compared with or without digestion. Mutant methyltransferase activity. Plasmid DNAs carrying wild-type M.Hhal or the Gly-81 mutant were much less viable and no survivors at the final time point in Figure 1. This is similar to the case of M.EcoRII where a Gly mutant in the P-C motif was cytotoxic (13).

**Table 1. Activities of methyltransferases**

<table>
<thead>
<tr>
<th>Methyltransferase</th>
<th>$^{3}$H (cpm)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-81 (wt)</td>
<td>92,125</td>
<td>+/- 814</td>
</tr>
<tr>
<td>Arg-81</td>
<td>378</td>
<td>+/- 61</td>
</tr>
<tr>
<td>Gly-81</td>
<td>426</td>
<td>+/- 82</td>
</tr>
<tr>
<td>His-81</td>
<td>491</td>
<td>+/- 75</td>
</tr>
<tr>
<td>Ser-81</td>
<td>463</td>
<td>+/- 83</td>
</tr>
<tr>
<td>none</td>
<td>410</td>
<td>+/- 65</td>
</tr>
</tbody>
</table>

Cys-81 is the wild-type M.Hhal. The proteins were purified using the procedure described previously (16) and assayed for methyltransferase activity using bacteriophage lambda DNA, digested with HindIII, as substrate. Methylation was monitored by the transfer of the radiolabeled methyl group from $^{3}$H-labeled methyl group to the DNA. Results were averaged from three experiments.

Table 2. Properties of methyltransferases

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>M.Hhal (wt)</th>
<th>M.Hhal (Cys-81)</th>
<th>M.Hhal (Ser-81)</th>
<th>M.Hhal (His-81)</th>
<th>M.Hhal (Arg-81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arg</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ser</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Protein purification and DNA binding activity

The purified methyltransferases were tested for their ability to bind, in a gel shift assay, to a synthetic duplex 37-mer oligonucleotide containing the recognition sequence GGCG (Oligos 1 and 3). Although the Ser-81, His-81 and Arg-81 mutants were enzymatically inactive, they can bind to the duplex 37-mer as efficiently as does wild-type M.Hhal containing Cys-81.

**Figure 3. Gly mutant protein binding properties.** Gel mobility shift assays were carried out as described in Materials and Methods using 27 nM methyltransferase and 81 nm DNA. Panel A: Mutant (Gly-, His-, Arg-, Ser-81) or wild-type (Cys-81) M.Hhal were purified with the standard procedure (16) and assayed for binding to a DNA duplex containing the recognition sequence GGCG (oligos 1 and 2). Panel B: The Gly-81 mutant methyltransferase was purified from cells co-expressing M.Ss1 and assayed for binding as in Panel A. Wild-type M.Hhal (Cys) is shown for comparison. Panel C: As Panel B, except that the oligonucleotide contained the sequence CCGG in place of GGCG (oligos 5 and 6).
Figure 4. Dissociation of the DNA-methyltransferase complexes. A. The Gly-81 mutant protein, Gly(s), was purified from E.coli coexpressing M.Ssrl. 40 nM wild-type M.HhaI (Cys) or 3 nM Gly-81 methyltransferase were incubated with 50 nM radiolabeled duplex probe containing the GCGC target sequence (oligos 1 and 3). The protein-DNA complex was then incubated with a 100 fold excess of unlabeled probe for different times. The amount of oligonucleotide bound to protein at different times was quantitated using a Phosphor-imager (Molecular Dynamics). The data for the Arg-81, His-81 and Ser-81 mutants are not shown, but were used to plot the results seen in panel B. The protein and oligonucleotide concentrations were such that binding was within the linear range. B. The time courses for dissociation of protein-DNA complexes between the wild-type M.HhaI (Cys) or the various mutants at position 81 (Arg, Gly, His, Ser) and a duplex oligonucleotide containing the recognition sequence (oligos 1 and 3) were calculated and normalized with respect to the total bound complex before the addition of competitor oligonucleotide.

Figure 5. Equilibrium binding of various methyltransferases to DNA. A. Wild-type M.HhaI (Cys) or mutants at position 81 (His, Gly), at the concentrations indicated, were incubated for 30 min at 22°C in a binding reaction that also contained SAH and the labelled 37-mer duplex DNA (oligos 1 and 3). Separate controls (not shown) established that in each case the binding reached equilibrium under the conditions used. Samples were analyzed by electrophoresis on a 10% native polyacrylamide gel. The gel was dried and the bands quantitated using a Phosphor-imager. B. The Kd values for wild-type and mutant M.HhaI were determined by Scatchard analyses. r = bound DNA probe/total input protein. [S]f = free DNA probe.
showed that it was a stronger DNA binding protein than the wild-type.

To purify the Gly-81 mutant free of DNA we co-expressed it in the presence of M.SssI, which methylates all CG sequences. We anticipated that M.HhaI and its Gly-81 mutant would probably bind very poorly to pre-methylated DNA, the product of the normal methylation reaction and so might be available in free form from this strain. Cells harboring both the M.SssI and Gly-81 mutant methyltransferase grew more slowly than cells expressing a single methyltransferase. This phenomenon also occurred in cells coexpressing the wild-type M.HhaI and M.SssI methyltransferases (Fig 1) and in cells expressing M.SssI alone (data not shown). This slower growth is probably due to methylation by M.SssI of sites required for normal cell growth because M.SssI was constitutively expressed in the cells. It proved easy to purify the Gly-81 mutant protein, free of DNA, from this strain and the purified protein was then proficient for DNA binding (Figure 3). The observed binding was not due to M.SssI contamination since no shifted complex was seen when an oligonucleotide containing the sequence CCGG was used.

The Kₐ values (Table 2) indicate that the Gly-81 mutant has a higher affinity for its target DNA than the wild-type methyltransferase. The kₐff values further (Table 2) substantiate this result. Because of the inherent difficulties in measuring accurate Kₐ values using gel shift assays the absolute values reported here should be treated with caution. There appears to be an inverse correlation between the sidechain length of the amino acid at position 81 and the stability of the DNA-protein complex. It is likely that a bulky side chain creates steric problems during the initial interaction between the methyltransferase and its DNA substrate leading to low affinity for the Arg-81 and His-81 mutants. This will be reflected in increased kₐff values because of the instability of the interaction between the protein and its target sequence. In contrast, the Gly-81 mutant has no side chain and has the apparent effect of stabilizing the complex between the mutant methyltransferase and its DNA target. The results reported here are fully consistent with the role of the P-C motif as the active site of DNA m5C-methyltransferases (9,10).

We have already reported the successful crystallization of M.HhaI in complex with its cofactor S-adenosylmethionine (16). Unfortunately, it is not possible to obtain crystals of the wild-type M.HhaI in complex with both SAM and DNA, because such a complex would be competent for catalysis. However, it may be possible to obtain crystals between the Gly-81 mutant and DNA in the presence of SAM. Such a complex might be expected to resemble the wild-type complex, but would be catalytically inactive. This would give further insight into the reaction mechanism and would complement structural studies of both the

**Table 2. Kinetic constants for the methyltransferases**

<table>
<thead>
<tr>
<th>Methyltransferase</th>
<th>t_{1/2} (min)</th>
<th>k_{off}×10^{-3} (min⁻¹)</th>
<th>Kₐ×10^{-9} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-81 (wt)</td>
<td>11</td>
<td>65</td>
<td>1.25</td>
</tr>
<tr>
<td>Arg-81</td>
<td>1.5</td>
<td>481</td>
<td>11</td>
</tr>
<tr>
<td>Gly-81</td>
<td>568</td>
<td>1.2</td>
<td>0.45</td>
</tr>
<tr>
<td>His-81</td>
<td>0.63</td>
<td>1000</td>
<td>20</td>
</tr>
<tr>
<td>Ser-81</td>
<td>69</td>
<td>10</td>
<td>1.69</td>
</tr>
</tbody>
</table>

Cys-81 is the wild-type M.HhaI. t_{1/2} and k_{off} (values calculated as ln2/t_{1/2}) were from the data of Figure 4B. The Kₐ was calculated by Scatchard analysis from the data of Figure 5.

**DISCUSSION**

The DNA methyltransferases that form 5-methylcytosine share a common architecture and contain a motif with absolutely conserved proline and cysteine residues (termed the P-C motif) that forms part of their catalytic center. Mutations at this conserved cysteine residue in M.EcoRII result in the abolition of catalysis when Gly, Ser, Val or Trp are present (13) and a Cys to Ser mutation abolishes activity in the SPR methyltransferase (12). A mutant containing Gly at this position in M.EcoRII is cytotoxic because of enhanced DNA methylation by M.Hhal. t_{1/2} Cys-81 (wt) is 3.9 minutes while t_{1/2} Arg-81 and His-81 mutants bound 10-fold and 20-fold more tightly than the wild-type respectively.

First, we determined the effects of the mutants on cell growth. Cells expressing the Gly-81 mutant methyltransferase were much less viable than cells expressing the Arg-81, Ser-81 or His-81 mutants. This parallels results obtained in the M.EcoRII system (13). We suspected that expression of the Gly-81 mutant methyltransferase was cytotoxic because of enhanced DNA binding to the target GCCG sequence, which might be expected to interfere with DNA replication and/or transcription. Two lines of evidence support this idea. First, the Gly-81 mutant protein, purified from the usual *E.coli* host, was found to contain bound DNA that rendered it unable to bind in *vitro* to exogenously-added oligonucleotides containing the GCCG sequence. Second, *in vitro* binding studies of the Gly-81 mutant purified free of DNA containing the GCCG recognition sequence. Complexes were formed between labelled DNA and the methyltransferase and then an excess of unlabelled oligonucleotide was added. Under the conditions used only specific complexes were seen. The amount of complex remaining in the reaction was measured as a function of time using a gel mobility shift assay (Figure 4A) and the results were quantitated. The amount of oligonucleotide bound by protein at different times was calculated from Phosphor-Imager analysis. The t_{1/2}, the time required for half of the complex to dissociate, and K_{off}, were calculated from the data in Figure 4. The derived values for t_{1/2} and k_{off} are shown in Table 2. The t_{1/2} for the Gly-81 mutant was increased about 50-fold over wild-type.

The dissociation constants (Kₐ) for the specific interaction between the wild-type and mutant methyltransferases with an oligonucleotide duplex containing the recognition sequence was calculated from a Scatchard analysis of the gel mobility-shift data (Figure 5). For this analysis we used a fixed protein concentration and varied the input DNA concentration as indicated in Figure 5. Each complex was assumed to contain one molecule of protein bound to one molecule of DNA (19). The bound protein concentrations were calculated as the difference between the total input oligonucleotide concentration minus the free oligonucleotide concentration. The Kₐ values obtained from this analysis, based on Kd = [S][P]/[SP], are shown in Table 2. It can be seen that the Gly-81 mutant binds DNA 3-fold more tightly than the wild-type methyltransferase or the Ser-81 mutant. In contrast, the Arg-81 and His-81 mutants bound 10-fold and 20-fold more weakly than wild-type respectively.

"Nucleic Acids Research, 1993, Vol. 21, No. 10 2463"
M.Hhal-SAM complex, which is almost complete, and that of a complex between M.Hhal, S-adenosylhomocysteine and DNA, which is currently in progress (S. Klimasauskas, R.J. Roberts and X. Cheng, submitted for publication).

ACKNOWLEDGMENTS

We thank Saulius Klimasauskas and Stefan Stamm for their help in calculating kinetic constants, Karen McClay for technical assistance and Tom Melendy, David Barford, Stefan Stamm, Eric Chang and Vincent Jung for helpful comments on the manuscript. We especially thank Ashok Bhagwat for his suggestion that prior methylation might be helpful in purifying the Gly-81 mutant protein. We thank J. Duffy and P. Renna for art and photography. S.M. is a recipient of an American Cancer Research Postdoctoral 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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