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

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ABSTRACT

The *Hha*I 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 6-position of cytosine prior to methyl group transfer. For the *Eco*RII methyltransferase, it has been shown that substitution of this catalytic cysteine by glycine is cytotoxic to *E.coli* cells expressing the mutant methyltransferase (Wyszynski et al. *Nucl. Acids Res.* 20: 319, 1992). We now show that this observation can be extended to the *Hha*I 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 (m^5C-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 m^5C-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 *Hae*III 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, more extensive study of the *Eco*RII methyltransferase (M.*Eco*RII, 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 *Hha*I methyltransferase (M.*Hha*I, 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_{off} about 50-fold. This enhanced affinity for target DNA probably interferes with cellular DNA replication or transcription causing cytoxicity.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K-12 strains: ER1727 [Δ(mcrB-mrr)102::-Tn10, mcrA1272::Tn10, F′ lac proAB $lacI^qΔ$(lacZ)M15] was kindly provided by E. Raleigh. Plasmid pHSH0-1, which encodes the H0 methyltransferase (5), contains a mutant form (I2L) of M.*Hha*I under the control of the T7 promoter. The properties of this mutant are indistinguishable from wild-type (5). Plasmid pAIT contains M.*Sss*I and was kindly provided by W.Jack (New England Biolabs). Plasmid pGEM-3Zf(+), containing the f1 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. Deoxycytidine-5′[α-^{32}P]-triphosphate and deoxyadenosine-

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5'[α-^{32}P]-triphosphate (6000 Ci/mmole) 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[methyl-^{3}H]methionine (15 Ci/mmol) was from Amersham.

Site-directed mutagenesis

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

A: TGTGCAGGGTTTCCGCACCAAGCGTTT
B: GCAGGGTTTCCGVGCCAAGCGTTT

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(+)*Hha*I was constructed by subcloning the *Sph*I-*Hin*dIII fragment containing the whole reading frame of the M.*Hha*I gene from plasmid pHSH0-1 into pGEM3Zf(+). Plasmids pGEM3Zf(+)M.*Hha*I-His81, -Ser81, -Gly81 and -Arg81 contain mutations of Cys81 made by oligonucleotide directed mutagenesis (14) using oligonucleotides A or B. Mutations were confirmed by dideoxy 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 *Sph*I-*Hin*dIII fragment from pGEM3Zf(+)M.*Hha*I-Gly-81, Ser-81, Arg-81 and His-81, respectively, into identical sites within plasmid pUHE25.

Purification of M.*Hha*I and mutants

The Arg-81, Ser-81 and His-81 mutants of M.*Hha*I 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.*Sss*I, were cotransformed into ER1727 cells and selected using ampicillin and kanamycin. Cells were grown to an OD_{600} of 0.4, then induced by adding IPTG to a final concentration of 400 μg/ml. The cells were harvested after induction for 2 hrs. The cell pellet was resuspended in 10 mM HEPES (pH 7.2), 50 mM NaCl, 5 mM Na_2EDTA, 0.1% 2-mercaptoethanol and 10% glycerol. The crude cell extract was prepared using a French press (1200 psi) and precipitated by 20% $(NH_4)_2SO_4$. The pellet was resuspended in 20 mM Tris.HCl (pH 8.0), 50 mM NaCl, 5 mM Na_2EDTA, 0.1% b-mercaptoethanol and 10% glycerol. The protein was dialyzed against the same buffer to remove $(NH_4)_2SO_4$. Dialyzed material was applied to a Pharmacia MONO-Q FPLC column (HR5/5, Anion exchanger) using a flow rate of 0.5 ml/min. The protein was eluted at approximately 110 mM NaCl using a 20 ml linear gradient of NaCl (100−400 mM). In each case the final preparations were 90−95% pure as judged by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining.

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 *Hha*I restriction endonuclease (R.*Hha*I). Typically 0.3 μg plasmid DNA was digested with 20 units R.*Hha*I 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.*Hin*dIII was incubated in the presence of [^{3}H-methyl]-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 Na_2EDTA, 5 mM b-mercaptoethanol, 65 μM [^{3}H]-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).

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 CGC TGA CCC ACA ACA TCC G -3'
2. 5'- GAC TGG TAC AGT ATC AGG ^{m}CGC TGA CCC ACA ACA TCC G -3'
3. 5'- TCG GAT GTT GTG GGT CAG CGC CTG ATA CTG TAC CAG T -3'
4. 5'- TCG GAT GTT GTG GGT CAG ^{m}CGC CTG ATA CTG TAC CAG T -3'
5. 5'- TGC AGT CGC GAT GCC GGG TCA CCT TGA GG -3'
6. 5'- GTC AGC GCT ACG GCC CAG TGG AAC TCC 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 5 and 6 are complementary 29-mers used to detect non-specific binding. Oligos 2 and 4 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-^{32}P]dATP and [α-^{32}P]dCTP with a specific activity of 6000Ci/mmol.

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 Na_2EDTA, 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), 2mM Na_2EDTA). 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.*Hha*I. 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

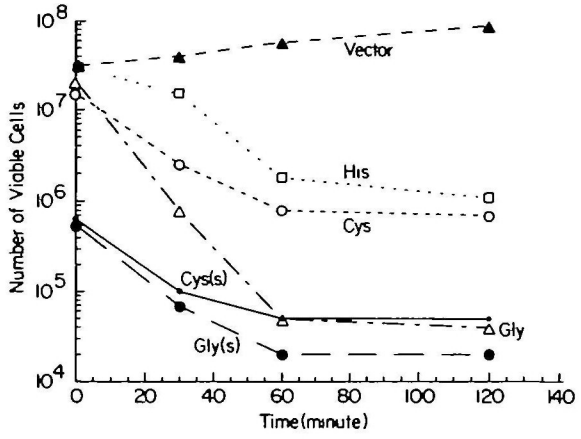

Figure 1. Effects of mutant methyltransferases on cell growth. Cells harboring expression plasmids for wild-type or mutant methyltransferases were induced by IPTG and then plated out at different times. Colonies were counted after 12 hrs and the colony forming units per ml was determined. Vector is the control plasmid without insert. Cys indicates wild-type M.*Hha*I containing Cys at position 81. His and Gly indicate mutants of M.*Hha*I containing His or Gly at position 81. Cys(s) and Gly(s) indicate the coexpression of wild-type M.*Hha*I or the Gly-81 mutant in the presence of M.*Sss*I.

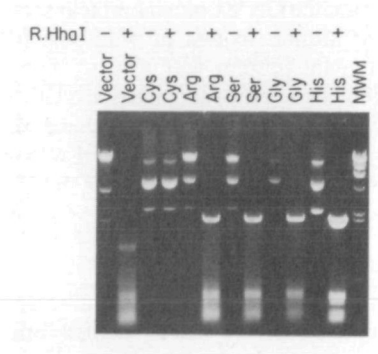

Figure 2. Mutant methyltransferase activity. Plasmid DNAs carrying wild-type or mutant methyltransferases were isolated and compared with or without digestion *in vitro* by *Hha*I restriction endonuclease (R.*Hha*I at top of figure). The digestion products were analyzed by 1% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide. The lane marked Vector contains plasmid DNA with no insert. Cys indicates the plasmid DNA expressing wild-type M.*Hha*I. Arg, Ser, His and Gly describe plasmid DNAs expressing the methyltransferase genes that carry the indicated mutations at position 81.

expressing the Arg-81, His-81 or Ser-81 mutants grew as well as cells expressing wild-type M.*Hha*I. The data (not shown) for the Arg-81 and Ser-81 mutants paralleled that for His-81. Cells expressing the Gly-81 mutant were much less viable and no plasmid DNA could be isolated from cells grown from the survivors at the final time point in Figure 1. This is similar to the case of M.*Eco*RII where a Gly mutant in the P-C motif was cytotoxic (13).

Mutant methyltransferase activities

Two tests were used to assay the activity of the mutant methyltransferases. First, plasmid DNAs from cells expressing the wild-type or mutant methyltransferase were isolated and their sensitivity to digestion by R.*Hha*I was determined *in vitro*. Active methyltransferases should methylate the plasmid *in vivo*, thereby protecting it from digestion *in vitro* by R.*Hha*I. Figure 2 shows

Table 1. Activities of methyltransferases

Methyltransferase	^{3}H (cpm)	Standard Deviation
Cys-81 (wt)	92,125	+/− 814
Arg-81	378	+/− 61
Gly-81	426	+/− 82
His-81	491	+/− 75
Ser-81	463	+/− 83
none	410	+/− 65

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

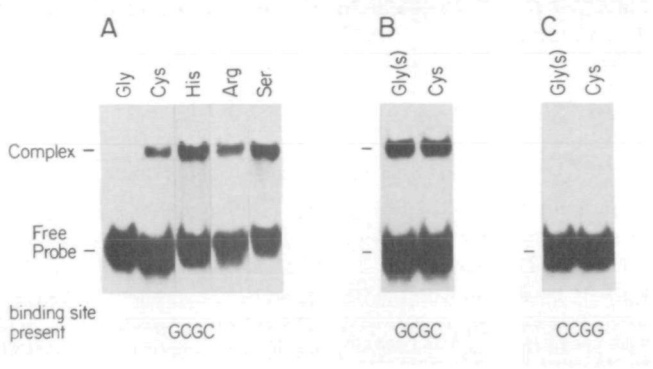

Figure 3. Gly mutant protein binding properties. Gel mobility shift assays were carried out as described in Materials and Methods using 27 nM methyltransferase and 81 nm DNA. **Panel A:** Mutant (Gly-, His-, Arg-, Ser-81) or wild-type (Cys-81) M.*Hha*I were purified by the standard procedure (16) and assayed for binding to a DNA duplex containing the recognition sequence GCGC (oligos 1 and 3). **Panel B:** The Gly-81 mutant methyltransferase was purified from cells co-expressing M.*Sss*I and assayed for binding as in Panel A. Wild-type M.*Hha*I (Cys) is shown for comparison. **Panel C:** As Panel B, except that the oligonucleotide contained the sequence CCGG in place of GCGC (oligos 5 and 6).

that plasmid DNA from cells expressing the wild-type methyltransferase gene was completely protected from R.*Hha*I digestion, whereas none of the plasmid DNAs expressing mutant methyltransferases were protected.

Second, the methylation activity of the wild-type and mutant methyltransferases was determined by measuring their ability to transfer [^{3}H]-labeled methyl groups from SAM to bacteriophage lambda DNA. Except for the Gly-81 mutant, the methyltransferases were purified close to homogeneity using a protocol similar to that developed for wild-type M.*Hha*I (16). No Gly-81 mutant was detected after the protamine sulfate precipitation step in the normal protocol. A modified procedure was used in which this step was replaced by a brief incubation with DNAse I. The final preparation was close to homogeneity as judged by SDS-PAGE. In contrast to the highly active wild-type enzyme, all of the mutants had activities close to background levels indicating that they were inactive as methyltransferases (Table 1).

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 GCGC (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.*Hha*I containing Cys-81

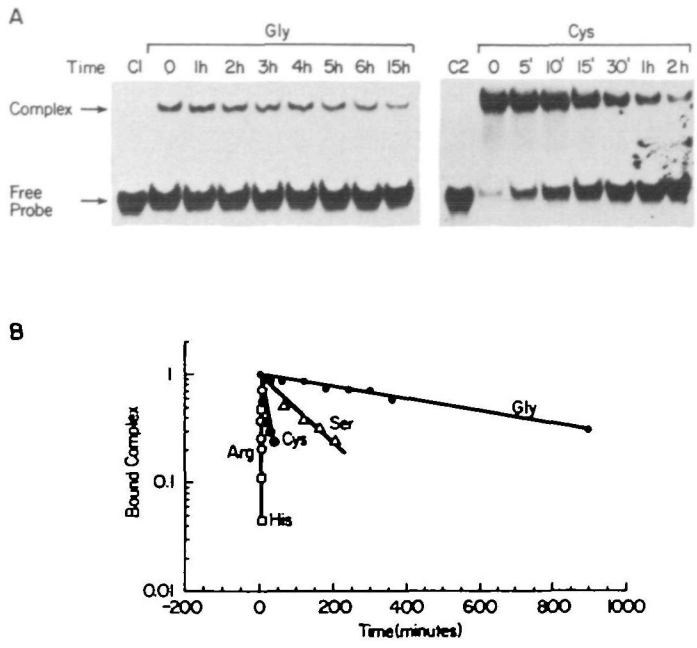

Figure 4. Dissociation of the DNA-methyltransferase complexes. **A.** The Gly-81 mutant protein, Gly(s), was purified from *E.coli* coexpressing M.*Sss*I. 40 nM wild-type M.*Hha*I (Cys) or 3 nM Gly-81 methyltransferase were incubated with 50 nM radiolabeled duplex probe containing the GCGC target sequence (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.*Hha*I (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 3). Unexpectedly, the Gly-81 mutant, purified by a modified version of the protocol used for the wild-type enzyme (16), showed no signs of DNA binding. However, the overall yield of this mutant methyltransferase was quite low. We considered the possibility that the Gly-81 mutant protein might in fact be a very strong DNA-binding protein and that we had actually purified only a small portion of it as a complex with DNA fragments. This was investigated by treating the purified wild-type or Gly-81 mutant proteins with T4-polynucleotide kinase and γ-P^{32}-ATP. Following the labelling, the mixture was extracted with phenol and the putative DNA products were analyzed by gel electrophoresis. A signal corresponding to labelled DNA was observed with the purified Gly-81 mutant, but not from a control preparation of purified wild-type M.*Hha*I (not shown).

To purify the Gly-81 methyltransferase free of DNA, we coexpressed the Gly-81 mutant in the presence of a plasmid encoding another methyltransferase, M.*Sss*I. M.*Sss*I forms 5-methylcytosine within the sequence CG (20) and would thus convert the M.*Hha*I recognition sequence, GCGC, into Gm5CGC. We anticipated that this prior methylation would block or weaken DNA binding by the wild-type M.*Hha*I and also of the Gly-81 mutant. This expectation was completely fulfilled. Purification of the Gly-81 mutant was greatly facilitated from a strain coexpressing M.*Sss*I (designated Gly(S) in Figures 1 and 3). In a gel shift assay, the Gly-81 mutant protein showed complex formation that was similar to that of wild-type M.*Hha*I. The purified Gly-81 mutant protein was able to bind the specific synthetic 37-mer (Oligos 1 and 3) containing the GCGC sequence, but not a control 29-mer (oligos 5 and 6) containing a CCGG sequence (Fig. 3, panels B and C). These results demonstrate that the binding is specifically contributed by the Gly-81 mutant methyltransferase, and is not a result of M.*Sss*I contamination.

Kinetics of dissociation

Dissociation rates were determined for the complexes between the mutant methyltransferases and a duplex oligonucleotide

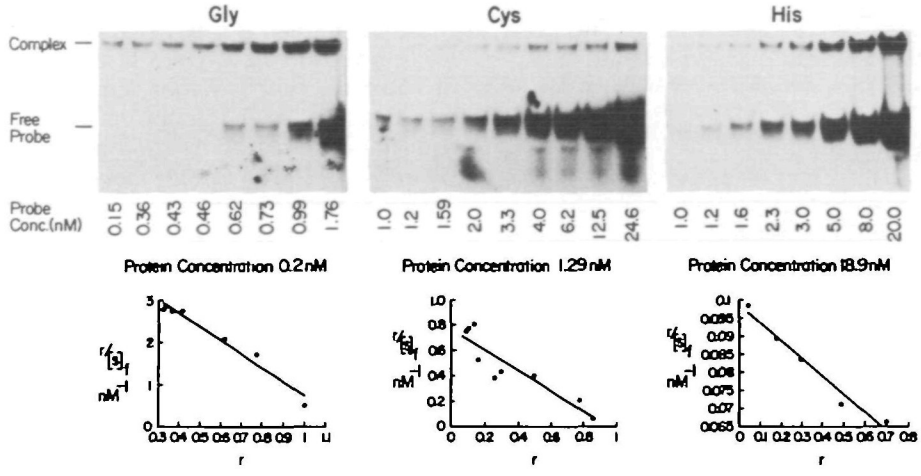

Figure 5. Equilibrium binding of various methyltransferases to DNA. **A.** Wild-type M.*Hha*I (Cys) or mutants at position 81 (His, Gly), at the concentrations indicated, were incubated for 30 min at 22°C in a binding reaction that also contained SAH and the labelled 37-mer duplex DNA (oligos 1 and 3). 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 K_d values for wild-type and mutant M.*Hha*I were determined by Scatchard analyses. r = bound DNA probe/total input protein. $[S]_f$ = free DNA probe.

containing the GCGC 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 (K_d) 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 K_d 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.

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.*Eco*RII 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.*Eco*RII is cytotoxic to *E.coli*. We were interested to know if this is a general phenomenon and, if so, to determine the cause of cytotoxicity since it might be informative about methyltransferase structure and function. We chose to study M.*Hha*I because this methyltransferase has recently been crystallized in our laboratory and its structure determination is in progress (16). Four mutants were generated by site-directed mutagenesis. The Cys-81 of the P-C motif was changed into either a Gly, Arg, Ser or His residue.

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.*Eco*RII system (13). We suspected that expression of the Gly-81 mutant methyltransferase was cytotoxic because of enhanced DNA binding to the target GCGC 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 GCGC sequence. Second, *in vitro* binding studies of the Gly-81 mutant purified free of DNA 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.*Sss*I, which methylates all CG sequences. We anticipated that M.*Hha*I 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.*Sss*I 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.*Hha*I and M.*Sss*I methyltransferases (Fig 1) and in cells expressing M.*Sss*I alone (data not shown). This slower growth is probably due to methylation by M.*Sss*I of sites required for normal cell growth because M.*Sss*I 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.*Sss*I contamination since no shifted complex was seen when an oligonucleotide containing the sequence CCGG was used.

The K_d values (Table 2) indicate that the Gly-81 mutant has a higher affinity for its target DNA than the wild-type methyltransferase. The k_{off} values further (Table 2) substantiate this result. Because of the inherent difficulties in measuring accurate K_d 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 k_{off} 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.*Hha*I in complex with its cofactor S-adenosylmethionine (16). Unfortunately, it is not possible to obtain crystals of the wild-type M.*Hha*I 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

Methyltransferase	$t_{1/2}$ (min)	$k_{off} \times 10^{-3}$ (min^{-1})	$K_d \times 10^{-9}$ M
Cys-81 (wt)	11	65	1.25
Arg-81	1.5	481	11
Gly-81	568	1.2	0.45
His-81	0.63	1000	20
Ser-81	69	10	1.69

Cys-81 is the wild-type M.*Hha*I. $t_{1/2}$ and k_{off} (values calculated as $\ln 2/t_{1/2}$) were from the data of Figure 4B. The K_d was calculated by Scatchard analysis from the data of Figure 5.

M.*Hha*I-SAM complex, which is almost complete, and that of a complex between M.*Hha*I, S-adenosylhomocysteine and DNA, which is currently in progress (S. Klimasauskas, R.J. Roberts and X. Cheng, submitted for publication).

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