"Inhibition of *Hha*I DNA (Cytosine-C5) methyltransferase by oligodeoxyribonucleotides containing 5-aza-2´-deoxycytidine: Examination of the intertwined roles of co-factor, target transition state structure and enzyme conformation." Brank, A.S., Eritja, R., Güimil García, R., Marquez, V.E., Christman J.K. J. Mol. Biol., 323(1), 53-67 (2002).
PMID: 12368098, doi: 10.1016/S0022-2863(02)00918-X

Running Title: Inhibition of M.*Hha*I by ZCyt ODNs.

Inhibition of *Hha*I DNA (Cytosine-C5) methyltransferase by oligodeoxyribonucleotides containing 5-aza-2'-deoxycytidine: Examination of the intertwined roles of co-factor, target, transition state structure and enzyme conformation.

Adam S. Brank[1], Ramon Eritja[3], Ramon Guimil Garcia [3], Victor E. Marquez [4]

and Judith K. Christman[1,2]*

[1] Department of Biochemistry and Molecular Biology and [2] UNMC/Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE, 68198-4525, USA;

[3] Centro de Investigación y Desarrollo, C.S.I.C., E-08034 Barcelona, Spain;

[4] Laboratory of Medicinal Chemistry, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702

*To whom correspondence should be addressed: Judith K. Christman;

Telephone: 402-559-6660;

Fax: 402-559-6650; jchristm@unmc.edu

SUMMARY

The presence of 5-azacytosine (ZCyt) residues in DNA leads to potent inhibition of DNA (Cytosine-C5) methyltranferases (C5-MTases) *in vivo* and *in vitr*o. Enzymatic methylation of cytosine in mammalian DNA is an epigenetic modification that can alter gene activity and chromosomal stability, influencing both differentiation and tumorigenesis. Thus, it is important to understand the critical mechanistic determinants of ZCyt's inhibitory action. Although several DNA C5-MTases have been reported to undergo essentially irreversible binding to ZCyt in DNA, there is little agreement as to the role of AdoMet and/or methyl transfer in stabilizing enzyme interactions with ZCyt. Our results demonstrate that formation of stable complexes between *Hha*I methyltransferase (M.*Hha*I) and oligodeoxyribonucleotides containing ZCyt at the target position for methylation (ZCyt-ODNs) occurs in both the absence and presence of co-factors, AdoMet and AdoHcy. Both binary and ternary complexes survive SDS-PAGE under reducing conditions and take on a compact conformation that increases their electrophoretic mobility in comparison to free M.*Hha*I. Since methyl transfer can occur only in the presence of AdoMet, these results suggest 1) that the inhibitory capacity of ZCyt in DNA is based on its ability to induce a stable, tightly closed conformation of M.*Hha*I that prevents DNA and co-factor release and 2) that methylation of ZCyt in DNA is not required for inhibition of M.*Hha*I.

KEY WORDS: DNA methyltransferase, methylation inhibitor, 5-azacytidine, gene activation, epigenetics.

INTRODUCTION

Enzymatic methylation of DNA at carbon 5 of cytosine has been shown to play a role in the regulation of a number of cellular processes through its ability to alter protein:DNA interactions. Changes in methylation status of specific regions of the genome have been implicated in the regulation of imprinting, maintenance of chromosome stability and altered gene expression in tumorigenesis (Baylin, 1997; Chen et al., 1998; Li et al., 1993). Although it is well documented that early alterations in the process of DNA methylation during development of tumors result in global hypomethylation of genomic DNA (Compans, 2000; De Marzo et al., 1999; Ehrlich et al., 1982; Goelz et al., 1985), a paradoxical hypermethylation of specific CpG islands has been linked to silencing of several genes involved in tumor suppression and mismatch repair (el-Deiry et al., 1991; Liang et al., 1998; Malumbres et al., 1999). In light of recent reports that some of these methylated genes can be reactivated by inhibitors of DNA methylation such as 5-azacytidine (ZCyd) and 5-aza-2'deoxycytidine (ZdCyd), and earlier studies showing that inhibition of methylation in certain tumor cell lines can induce differentiation (Christman et al., 1983; Creusot et al., 1982 and references in Zingg & Jones, 1997)), interest in the therapeutic uses of inhibitors of DNA methylation has been revived. ZCyd and ZdCyd have already been used as therapeutic agents for treatment of sickle cell anemia, myelodysplastic syndrome and several other cancers with varying degrees of success (Goldberg et al., 1993; Pinto & Zagonel, 1993; Rochette et al., 1994).

In cells, ZCyd and ZdCyd are converted to deoxyribonucleoside triphosphates and subsequently incorporated into newly synthesized DNA in place of cytosine residues. DNA C5-MTase is inactivated through binding to ZCyt residues that replace Cyt residues next to guanine (CpG), resulting in global hypomethylation of newly synthesized DNA (Christman, 1984; Creusot et al., 1982). Depending on the cell type, a variety of genes are activated and play a role in differentiation. Unfortunately, ZCyd is cytotoxic, mutagenic and unstable in aqueous solution (Chatterji & Gallelli, 1979; Jackson-Grusby et al., 1997; Juttermann et al., 1994; Vesely et al., 1968), which limits its value for patient treatment. This has led us to investigate the potential of small, defined oligodeoxyribonucleotides (ODNs) containing ZCyt as vehicles to inhibit intracellular DNA methylation with a less toxic form of the inhibitor. Theoretically, ZCyt-ODNs will act as specific inhibitors of DNA C5-MTases that do not require metabolic activation and that do not have to be incorporated into the host genome to inhibit DNA methylation. The studies described here were initiated to determine the critical mechanistic determinants of the inhibitory capacity of small ZCyt-ODNs. M.*Hha*I, a well-characterized prokaryotic DNA C5-MTase was chosen as a surrogate for the larger, more complex mammalian enzymes because it has catalytic domains highly homologous to those of human and murine DNA C5 MTase and its interactions with small double stranded ODNs and co-factors have already been defined by X-ray crystallographic studies (Cheng et al., 1993; Klimasauskas et al., 1994; Kumar et al., 1994; O' Gara et al., 1998; O' Gara et al., 1996; Schluckebier et al., 1995; Sheikhnejad et al., 1999).

Previous reports describing the interactions of prokaryotic DNA C5-MTases, *Eco*RII methyltransferase (M.*Eco*RII) (Friedman, 1986; Gabbara & Bhagwat, 1995), *Msp*I methyltransferase (M.*Msp*I), M.*Hha*I and *Hpa*II methyltransferase (M.*Hpa*II) (Friedman, 1986; Santi et al., 1984) with DNA or ODNs containing ZCyt residues provided support for a

mechanism of inhibition involving formation of a covalent bond between the active site cysteine residue of the C5-MTase and C6 of ZCyt in DNA. However, the effect of co-factor on the formation of complexes containing DNA C5-MTases and DNA containing ZCyt in place of cytidine has been reported to vary depending on the enzyme. Binding of M.*Eco*RII and M.*Hha*I to ZCyt-containing DNA were reported to occur in the absence of co-factor and to be stimulated in the presence of co-factor (AdoMet, AdoHcy, or Sinefugin) (Friedman, 1986; Gabbara & Bhagwat, 1995). M.*Msp*I and M.*Hpa*II binding to ZCyt-containing DNA were reported to be dependent on the presence of co-factor (AdoMet, Sinefugin, and AdoHcy) (Friedman, 1986). However, in separate reports, M.*Hpa*II and murine C5-MTase binding to ZCyt-containing DNA was reported to be equally effective in the absence or presence of AdoMet (Christman et al., 1985; Santi et al., 1984). It remains to be determined whether the involvement of co-factor in these complexes is due to inherent differences in the enzymes, differences in substrates (biologically synthesized DNA with random substitution of ZCyt, or synthetic substrates with ZCyt in defined sites), or method of assay. It is also unclear whether the transfer of methyl groups to ZCyt in DNA is a common feature of DNA C5-MTases. Thus far, there has been only one report indicating that M.*Eco*RII can transfer methyl groups to ZCyt residues in DNA (Gabbara & Bhagwat, 1995). Earlier studies did not measure methylation directly, but it was inferred that methylation was not required for most DNA C5-MTases because complex formation occurred in the absence of methyl donor, AdoMet, and in the presence of AdoHcy, an AdoMet analog that is incapable of serving as a methyl-donor and acts as a competitive inhibitor of AdoMet binding (Christman, 1984; Friedman, 1985; Friedman, 1986; Gabbara & Bhagwat, 1995).

The study reported here is unique in that we have used small ODNs in which a ZCyt residue replaces the single target cytosine (ZCyt-ODN) in a hemi-methylated GCGC site (see Table 1 for ODN sequences). We have assessed the effects of co-factor interactions on complex stability not only by native gel electrophoresis, but also by gel-exclusion chromatography and SDS-PAGE of complexes subjected to heating in the presence of SDS and reducing agent. Our results sugget that, even though M.*Hha*I can methylate ZCyt-ODNs when AdoMet is present, only a minor fraction of ODNs in these extremely stable ternary complexes is methylated. The importance of co-factor in the formation and stabilization of the M.*Hha*I:ZCyt-ODN complex was examined in detail. Evidence is presented demonstrating that the interaction of M.*Hha*I with ZCyt-ODNs induces the same dramatic conformational change in the enzyme in the presence or absence of co-factor. However, while co-factor (AdoHcy>AdoMet) was found to significantly stabilize M.*Hha*I:ZCyt-ODN complexes under non-denaturing conditions, once the complexes were formed, cofactor had no effect on their thermal stability when heated under denaturing conditions. The importance of these results to understanding the inhibitory mechanism of ZCyt residues in DNA is discussed.

RESULTS

Inhibition of M.*Hha* I by ZCyt-ODNs. The small hemimethylated ODNs that were used in this study (Table 1) have already been shown to act as inhibitors of M.*Hha*I when 5-fluorocytosine (FCyt) is substituted for the target Cyt (underlined) in the M.*Hha*I recognition sequence 5'-GCGC-3' (Sheikhnejad et al., 1999). To establish the effectiveness of ZCyt-ODNs as inhibitors of methylation by M.*Hha*I, the relative inhibitory potency of ODNs containing ZCyt or FCyt in place of the target cytosine was compared. Increasing concentrations of AMp:A' substrate and

the indicated concentrations of inhibitor (15-120 nM) were added to a standard reaction mixture containing 8.6 nM M.*Hha*I. The rate of incorporation of radiolabeled methyl groups from [*methyl*-^{3}H]AdoMet was determined during a 5 minute incubation (See Materials and Methods). It can be seen (Figure 1, A, B), that both sets of data intersect at the X-axis in a Lineweaver-Burk plot, indicating a non-competitive form of inhibition, a result consistent with either covalent bond formation or high affinity non-covalent binding between M.*Hha*I and the inhibitor ODNs. The apparent Ki values derived from these data were 200±73 nM for FCyt-ODNs (AFp:A'Mp) and 4.3±0.65 nM for ZCyt-ODNs (BZ_7:B'M_6) indicating stochiometric formation of inhibitory complexes between ZCyt-ODNs and M.*Hha*I during a 5 minute reaction. While these Ki's suggest that M.*Hha*I:ZCyt-ODNs are at least 50-fold more potent as inhibitors than FCyt-ODNs, such calculations are only accurate if all components of reaction are in equilibrium. Since equilibrium cannot be reached when the inhibitor is covalently bound to the enzyme or when the rate of dissociation of the inhibitor from the enzyme is essentially zero during the reaction period, the Ki was determined by incubating 0.4 μM M.*Hha*I with increasing amounts of ZCyt- or FCyt-ODN for 0-5 minutes prior to addition of substrate. Using this method, the Ki for ZCyt-ODN was 0.7±0.07 μM with a k_{inact} of 1.6±0.06 (min $^{-1}$) compared to a Ki for FCyt-ODN of 1.0±0.09 with a k_{inact} of 0.78±0.07 (min $^{-1}$). Since the Ki's of ZCyt-ODN and FCyt-ODN are essentially the same and the rate of inactivation of M.*Hha*I is only ~2 fold faster with ZCyt-ODN than with FCyt-ODN, these results indicate that an additional factor, the affinity of binding the inhibitory ODN relative to that of the normal substrate, accounts for the much higher apparent inhibitory capacity of ZCyt-ODN when added to enzyme concurrently with an excess of substrate (Figure 1).

Formation and dissociation of M.*Hha* I:ZCyt-ODN complexes. Native gel shift assays were used to evaluate the influence of cofactors on the interaction between M.*Hha*I and ZCyt-ODN and to test the previously reported findings that DNA C5-MTases bind very tightly (or are covalently linked) to ZCyt residues in DNA (Christman et al., 1985; Friedman, 1985; Gabbara & Bhagwat, 1995; Santi et al., 1984). ZCyt-ODN (BZ_7:$B'M_6$, with ^{32}P-radiolabel at the 5'end of $B'M_6$) was incubated with M.*Hha*I in the presence or absence of co-factors for 30 minutes at 22 ^{o}C to allow formation of complexes. To determine whether the rate of dissociation of these binary and ternary complexes differed, 100-fold molar excess of unlabeled ZCyt-ODN was added to the reaction mixtures after initial complex formation, and the incubation was continued for up to 24 h as indicated. Complexes were then separated from unbound ZCyt-ODNs by electrophoresis on non-denaturing 10% polyacrylamide gels (details are given in Materials and Methods and the caption for Figure 2, where the results of a typical experiment are presented). It can be seen that the presence of AdoMet in the reaction mixture increased the amount of M.*Hha*I:ZCyt-ODN complex formed (% Bound) compared to reaction mixtures containing AdoHcy or without co-factor (Figure 2, compare Lane 6 to Lanes 1 or 11). However, it is clear that in all the reactions more than 85% of the input radiolabeled ZCyt-ODNs were bound in binary (- co-factor) or ternary complexes (+AdoHcy, +AdoMet) in the presence of non-specific competitor DNA (poly dAdT:dTdA) (Lanes 1, 6, and 11). Addition of specific competitor (100 fold-excess unlabeled ZCyt-ODN) caused an immediate decrease in the amount of bound radiolabeled ODN. The extent of this decrease was greatest for complexes formed with AdoHcy or in the absence of co-factor (32%, for Lanes 1,2 and 31% for lanes 11,12). In the presence of AdoMet, approximately 20% of bound radiolabeled ODN was displaced (lanes 6 and 7). The primary source of these displaced ODNs was a "smear" of complexes that were present in all

binding reactions that migrated more rapidly than the major band of complexes. A biphasic pattern of dissociation was observed for the remaining radiolabeled complexes. During the first phase, the rate of dissociation of both binary and ternary complexes was approximately the same, i.e., a plot of dissociation data from phase I (Figure 3 and Table 2) predicts a $t_{1/2}$ of 1-1.4 h for all complexes. However, during phase II (dissociation from 2-30 hrs) the effect of co-factor on dissociation becomes obvious (Figure 3 and Table 2). The rank order for co-factor stabilization of M.*Hha*I: ZCyt-ODN complex was: AdoHcy>AdoMet>no co-factor. The data from phase II indicated that complexes without co-factor dissociated with $t_{1/2}$ values of approximately 4.5 days while the estimated $t_{1/2}$ for complexes containing co-factor was anywhere from 13 days (AdoMet) to >3 weeks (AdoHcy). The results for binary complexes and ternary complexes with AdoMet are similar to those reported by Santi et al. (1984) who found no significant decrease in the amount of M.*Hpa*II:ZCyt DNA complexes formed in the presence or absence of AdoMet during three days incubation in the presence of 30-fold cold competitor. Thus, extremely stable complexes between M.*Hha*I and ZCyt in DNA are indeed possible in the absence of AdoMet and their stability is greatest in the presence of the methylation inhibitor AdoHcy. This strongly supports the hypothesis that the mechanism of M.*Hha*I inhibition by ZCyt-containing DNA does not require methylation. Since it has been demonstrated that hydrolysis of ZCyd in solution leads to ring-open forms which can undergo further degradation (Beisler, 1978), we compared the dissociation of M.*Hha*I:ZCyt-ODN and M.*Hha*I:AP-ODN complexes (AP = abasic furanose target) to determine the effect of complete absence of a normal base on binding. AP-ODNs, which can neither be methylated nor form covalent bonds with C5-MTases, form surprisingly stable complexes with M.*Hha*I even in the absence of co-factor (Table 2) but have a monophasic dissociation curve (Data not shown). The distinct difference between the patterns and rates of

dissociation of M.*Hha*I:AP-ODN and M.*Hha*I:ZCyt-ODN complexes indicates that neither the rapidly nor the slowly dissociating M.*Hha*I:ZCyt-ODN complexes have undergone complete loss of the ZCyt pyrimidine base. In phase II of dissociation, M.*Hha*I complexes formed with ZCyt-ODNs in the absence of co-factor have similar $t_{1/2}$ values to complexes formed with AP-ODNs. However AP-ODN complexes are much less sensitive to the stabilizing effect of co-factor. For example, AdoHcy increases the $t_{1/2}$ for M.*Hha*I:ZCyt-ODN complexes >10 fold while the $t_{1/2}$ for M.*Hha*I:AP-ODN complexes is increased <4 fold in the presence of AdoHcy. As will be discussed in greater detail below, this suggests that the interaction between the base and co-factor in complexes is the primary influence on the stability of enzyme:DNA complexes.

Binding to ZCyt-ODN induces a stable conformational change in M.*Hha* I. In ternary complexes with FCyt-ODNs, the target FCyt is methylated and covalently linked to M.*Hha*I. The covalent bond in FCyt-ODN ternary complexes is stable to heating at 95 ^{o}C in the presence of 1% SDS and β-mercaptoethanol, while ZCyt-ODN complexes are completely dissociated under these conditions (Figure 4). This dissociation occurs in the absence of any scission of ZCyt-ODNs at 95 ^{o}C, either in the presence or absence of enzyme (Figure 5). To examine the stability of ternary complexes containing ZCyt-ODNs in greater depth, we compared FCyt- and ZCyt-ODN ternary complexes using SDS-PAGE analysis. The complementary strand of the ds ODN (Table 1, A' or B' strand), which contains MCyt in the M.*Hha*I target base position, was ^{32}P-radiolabeled on the 5' end to allow simultaneous detection of both the ODN and M.*Hha*I. In the absence of ODN, the electrophoretic mobility of M.*Hha*I after SDS-PAGE was identical regardless of whether the enzyme was heated to 95 ^{o}C or maintained at 22 ^{o}C in the presence of SDS and β-mercaptoethanol prior to electrophoresis (Figure 4A, Coomassie blue stained gel,

Lanes 1, 2). Incubation of M.*Hha*I with FCyt-ODN and AdoMet at 37 ^{o}C for 1 h, followed by treatment with SDS and β-mercaptoethanol at 22 ^{o}C did not appear to alter its migration from that of the free enzyme when examined by Coomassie blue staining (Figure 4A, Lane 4). However, the distribution of complexes containing ^{32}P-FCyt-ODN (Figure 4B, Lane 4) suggests that the majority of the ternary complexes were distributed over a broad area of the gel lane (Apparent molecular mass 33-59 KDa). Thus, most of the enzyme had not formed stable complexes with FCyt-ODN during the incubation period. After heating to 95 ^{o}C, the intensity of Coomassie blue staining of the free protein band was increased and a slower migrating band with the mobility predicted for a complex containing one molecule of FCyt-ODN (AFp:A'Mp) covalently bound to one molecule of M.*Hha*I (~59 KDa) appeared along with an array of protein bands of intermediate mobility (Figure 4A, Lane 3). Only the slowest migrating band contained ^{32}P-FCyt-ODN (Figure 4B, Lane 3). In contrast, ternary complexes of M.*Hha*I with ^{32}P-ZCyt-ODN actually migrated more rapidly than free enzyme after treatment with 1%SDS and β-mercaptoethanol at 22 ^{o}C (Compare Figure 4, Panel A, B-Lanes 6, 7 with Panel A-Lanes 1, 2). As estimated from the intensity of Coomassie blue staining, more than 50% of the input enzyme protein was present in a rapidly migrating complex with ^{32}P-ZCyt-ODNs (Figure 4, Panel A, B, Lane 7). This complex, which has an electrophoretic mobility consistent with a 30 kDa protein, persists even after heating to 70 ^{o}C for 5 minutes prior to electrophoresis (Figure 4, Panel A, B, Lane 6). However, when an aliquot of the same reaction mixture was heated to 95 ^{o}C, all of the protein migrated with the mobility expected for completely denatured M.*Hha*I (Panel A, Compare Lane 6 and Lane 2) and the ODN migrated with the mobility expected for the free ODN (Panel B, Lane 6). Thus, it is clear that the higher mobility complex is not simply the result of enzyme degradation during the reaction with ZCyt-ODNs.

These experiments indicate that complexes involving M.*Hha*I and ZCyt-ODNs that are stable in the presence of SDS and β-mercaptoethanol migrate with a higher mobility in denaturing SDS gels because of a reversible change in the conformation of M.*Hha*I that results from its interaction with the ZCyt target. The mobility of this complex differs markedly from that of the complex resulting from M.*Hha*I methylation of and covalent linkage to an FCyt-ODN. Without heating, only a small proportion of the ternary M.*Hha*I:^{32}P-FCyt-ODN:AdoMet complexes migrate more rapidly than free enzyme (Figure 4, Panel B, lane 4), while heating at 95 ^{o}C converts all of the covalently linked complex to a low mobility form that migrates at the rate expected for M.*Hha*I covalently linked to FCyt-ODN.

ZCyt-ODN induction of M.*Hha*I conformational change is independent of co-factor. To further characterize the novel conformation of M.*Hha*I complexed to ZCyt-ODN, we compared the stability of M.*Hha*I:ZCyt-ODN complexes formed in the presence of AdoMet, AdoHcy or in the absence of co-factor. Complexes maintained at 22 ^{o}C were found to have the same electrophoretic mobility in both native and denaturing gels regardless of whether co-factor was present or not (Figure 2; Figure 6, Lanes 3, 6, 9). When reaction mixtures were heated to 70 ^{o}C prior to SDS-PAGE, complexes containing AdoMet or AdoHcy remained intact although the proportion of bound/free ZCyt-ODN in the reaction mixture was somewhat reduced. Under the same conditions, ZCyt-ODN was almost completely dissociated from complexes with enzyme alone (Figure 6, compare lanes 2 and 8 with lane 5). This result suggested that while co-factor is not a requirement for formation of high mobility M.*Hha*I:ZCyt-ODN complexes, the presence of co-factor does stabilize the complex against dissociation by heat in the presence of

1% SDS and β-mercaptoethanol. However, the rank order for amount of complex formed as detected in this assay was the same as that observed using native gel electrophoresis (AdoMet>AdoHcy>no co-factor). Thus, when the amount of complex remaining after heating to 50 and 75 ^{o}C was normalized to the amount of complex present after treatment with 1% SDS and β-ercaptoethanol at 22 ^{o}C (Figure 7), it became obvious that the effect of temperature on the rate of dissociation is similar whether co-factor is present or not, i.e., co-factor does not contribute significantly to heat stabilization of these compact high mobility complexes.

Detection of methylation of ZCyt-ODNs by M.*Hha* I. While the studies on the effects of cofactor on M.*Hha*I:ZCyt complex stability presented above clearly demonstrate that methyl transfer is not necessary for complex formation, they do not rule out the possibility that methyl transfer does occur during ternary complex formation in the presence of AdoMet. X-ray crystallographic analysis provided the final proof that when AdoMet is present, M.*Hha*I forms a covalent complex with FCyt in DNA and that carbon 5 of the target Cyt in the covalent complex carries both a fluorine and a methyl group (Klimasauskas et al., 1994). Even though transfer of this methyl group results in the inactivation of the enzyme, it is possible to directly measure methyl transfer to FCyt-ODN using high concentrations of enzyme and [*methyl*-^{3}H]AdoMet with a specific activity of ~3000 GBq/mmol (Sheikhnejad et al., 1999). The ability of M.*Hha*I to catalyze methyl transfer to FCyt- and ZCyt-ODN (BZ_7:B'M_6) was compared (Figure 8). As expected, FCyt-ODN was methylated during the process of covalent linkage to M.*Hha*I. However, at a ratio of 1.7 mol M.*Hha*I:1 mol ODN, (Fig 2, 100nM ODN) methyl transfer occurred at 37% of available FCyt targets but at only 2% of ZCyt targets. Since both substrates have a similar Ki, these results directly support the evidence provided by the binding assays

described above, i.e., that the inhibitory interaction between ZCyt-ODN and M.*Hha*I does not require methylation. Although is is not possible to rule out the possibility that some loss of radiolabeled, methylated ZCyt-ODN occurred during collection of the ODN by acid precipitation, no significant scission of ZCyt-ODN occurs during one hour incubation at 22 °C in the presence of 80% acetic acid or during acid precipitation (García et al., 2001) and data not shown. Furthermore, when *methyl*-^{3}H incorporation was determined using an alternative method carried out at neutral pH, binding of enzyme:ZCyt-DNA complexes to a nitrocellulose filter, followed by washing 0.3M NaCl and 0.1% Sarkosyl (Christman et al., 1985), radiolabel in M.*HhaI*:ZCyt-ODN complexes was not significantly greater than background while radiolabel in methylation in enzyme M.*Hha*I:FCyt-ODN complexes determined was comparable to that shown in Figure 8.

DISCUSSION

In 1984, Santi proposed that catalysis of methylation by DNA (cytosine-5)-methyltransferases was analogous to other enzyme-catalyzed transfers of one carbon units to C5 of pyrimidines, i.e., that the mechanism involves reversible formation of a dihydropyrimidine intermediate with a covalent linkage between the enzyme and C6 of the target Cyt and a carbanion equivalent at C5. This reactive intermediate could either 1) accept a proton from water or a general acid of the protein or 2) a methyl group from AdoMet. Abstraction of a proton from C5 followed by β-elimination of the enzyme nucleophile would lead to release of the DNA with either an (1) unmethylated or (2) methylated cytosine (Santi et al., 1984). He further proposed that covalent bonds formed with the Cyt analogs, FCyt and ZCyt, would not be easily reversible. Methylation

of FCyt or addition of a proton to N5 of ZCyt would lead to stable complexes with the enzyme covalently linked to the DNA substrate (See scheme for ZCyt, Figure 9, Structure IIb).

Formation of C5-MTase:FCyt-DNA complexes stable to heating at 95 ^{o}C in the presence of SDS and a reducing agent has since been reported by a number of investigators and the predicted FCyt intermediate with M.*Hha*I has been demonstrated in X-ray crystallographic studies (Klimasauskas et al., 1994). The hypothesis that covalent complexes occur with ZCyt-containing DNA is also supported by evidence of the formation of an essentially irreversible complex between ZCyt-substituted DNA and C5-MTases from bacterial and mammalian cells *in vitro* (Christman et al., 1985; Friedman, 1986; Gabbara & Bhagwat, 1995; Santi et al., 1984), and *in vivo* (Creusot et al., 1982; Flatau et al., 1984; Jackson-Grusby et al., 1997). However, as noted in the Introduction, there is little agreement regarding the role of co-factors and/or methyltransfer in complex formation and there is as yet no X-ray crystallographic data on the structure of ZCyt complexes with M.*Hha*I. Thus, the findings reported here provide several unique insights into the mechanism of methyl transfer and the probable structure of the M.*Hha*I:ZCyt-ODN complex.

Role of AdoMet in the formation and stabilization of ZCyt-ODN complexes. Although our data suggest that methylation of 1-5 % of ZCyt residues in our ODNs can occur, a rigorous determination of the extent to which ZCyt residues in DNA are methylated during complex formation with M.*Hha*I in the presence of AdoMet will require mass-spectrophotometric analysis. This is currently underway. The observation that methylation is not required for formation of stable complexes between ZCyt-ODN and M.*Hha*I is, however, consistent with Santi's proposal that addition of a proton at N5 can lead to formation of a stable but slowly

reversible covalent link between enzyme and ZCyt in DNA. Based on X-ray crystallographic and NMR studies, it has been proposed that protein-DNA complexes with at least three different structures are involved in the mechanism of action of M.*Hha*I. These include (1) an initial complex formed between the enzyme and a normally stacked B-DNA helix, (2) an "open" complex of enzyme and DNA comprised of "an ensemble of flipped out conformers", and (3) a more compact or closed complex in which the active site loop of the enzyme locks the flipped out target cytosine into the active site pocket (Klimasauskas et al., 1998). The "closed" configuration is manifested by a more rapid migration of ternary complexes with AdoHcy than for binary complexes in native polyacrylamide gels (Klimasauskas & Roberts, 1995; Sheikhnejad et al., 1999). Formation of "closed" complexes between M.*Hha*I and ODNs with Cyt or mismatched base (Ade, Ura) targets, as defined by native gel migration rates, are only detectable in the presence of AdoHcy (Klimasauskas & Roberts, 1995; Sheikhnejad et al., 1999). In contrast, ODNs containing 5,6-dihydro-5-azacytosine (DZCyt) or ZCyt targets form "closed" complexes with M.*Hha*I in both the presence and absence of co-factors (Figure 3 and Sheikhnejad et al., 1999). We have previously postulated that DZCyt, which cannot be methylated, either induces or allows a shift of equilibrium toward the "closed" M.*Hha*I conformation in the absence of co-factor because the shape of DZCyt in the catalytic pocket so closely mimics the shape and non-aromatic character of a dihydrocytosine reaction intermediate. This would be expected to reduce the energetic requirements for the massive conformational changes that occur when the target base is locked into the catalytic pocket, as it is when crystallized in the presence of AdoHcy (Klimasauskas & Roberts, 1995; Sheikhnejad et al., 1999). If the non-aromatic character of the target base is not only sufficient but also necessary for formation of "closed" complexes in the absence of co-factor, the results presented here

would support the hypothesis that formation of covalent complexes between M.*Hha*I and ZCyt does not require co-factor. Our results indicate that co-factor can, however, affect the rate of formation and stability of the complexes.

When native gel electrophoresis is used to quantitate binding of ZCyt-ODN to M.*Hha*I during a short incubation (Figure 3), it is consistently observed that the presence of AdoMet increases the amount of closed complex formed by >30% relative to the amount of closed complex formed in the presence of AdoHcy or in the absence of co-factor. However, the primary effect of the S^{+}-CH_3 moiety of AdoMet seems to be on rate of formation or initial stabilization of the complex rather than on its long-term stability. AdoHcy has a much greater effect on complex stability than AdoMet, increasing the $t^{1/2}$ for dissociation 10-fold relative to that found in the absence of co-factor. AdoMet only causes a 3-fold increase (Table 2).

There is some probability that at least three different forms of ZCyt are present in the ODNs used in our binding studies (Figure 9): Intact ZCyt (Structure I), the initial hydrolysis product of ZCyt which can be either in a non-aromatic ring or a ring open form (Structures IIa, IIIa), and the ring open form that has undergone loss of the formyl group (Structure IVa). Analysis by mass spectrometry revealed only two alternate forms of the ODN, a small amount of ODN with loss of 10 mass units, as would be predicted for an ODN with the ring open form following loss of the formyl group (Structure IVa) and a small amount of ODN missing 290 mass units, the mass of an ODN completely missing the ZCyt nucleotide (Eritja, 1997; García et al., 2001). The ODNs employed in the studies reported here were purified by ion exchange chromatography and no longer contain detectable amounts of the single nucleotide deletion ODN

(See Figure 5). Thus, it is most probable that the first phase of dissociation (Figure 4, Table 2) involves complexes formed between M.*Hha*I and ODNs containing the ring-open form IVa (ZCyt-IVa-ODN) in the target position. This proposal is supported by two aspects of the experimental data for phase I dissociation. The first aspect is the $t^{1/2}$ for dissociation. While the possibility exists that several of the structural features of the cytosine ring that interact with amino acids in the catalytic pocket of M.*Hha*I are maintained in ZCyt-IVa-ODN, i.e. the N3,N4 interactions with Glu^{119} , the O2 and phosphodiester backbone interactions with Arg^{165} and the N3 amino group interaction with Pro^{80} (O' Gara et al., 1996), ZCyt-IVA ODN has lost any possibility of stabilization through formation of a partial or complete covalent bond between C6 of ZCyt and Cys^{81} of M.*Hha*I. In addition, formation of an alternate ring structure through intramolecular hydrogen bond formation (Structure V) could interfere with enzyme/DNA interactions such as the one between O2 and Arg^{165}. Thus, it is not surprising that the flipped target base of ZCyt-IVA-ODN would behave similarly in its interactions with amino acids in the catalytic pocket to a non-cytosine base that cannot be methylated. Under conditions comparable to those employed here, $t^{1/2}$ for dissociation of ODNs with Ade and Ura targets are in 15-90 minutes range (Sheikhnejad et al., 1999 and unpublished A.S.B. and J.K.C.) while the $t^{1/2}$ determined for ZCyt-ODNs in phase I ranges from 60-80 minutes. The second aspect is the relatively small stabilizing effect of co-factors on rapidly dissociating Phase I M.*Hha*I:ZCyt-ODN complexes. This is similar to the observed behavior of binary complexes between M.*Hha*I and ODNs with Ade or Ura targets. These ODNs are in the open conformation and are only stabilized in the closed conformation by AdoHcy, not by AdoMet (Klimasauskas & Roberts, 1995; Sheikhnejad et al., 1999 and unpublished A.S.B. and J.K.C.). Typically, the degree of stabilization observed with the shift from open to closed complex with these mismatched bases is

at least 5-fold less than that observed with ODNs having Cyt or Cyt analogs as the target base. The Phase I ternary complexes with ZCyt-ODN are stabilized by no more than 50% relative to Phase I binary complexes. Careful examination of the native gel shift assays shown in Figure 3 also indicate that a small proportion of binary complexes migrates with the slower rate characteristic of open complexes. These complexes do not dissociate immediately upon addition of cold competitor but are completely dissociated within 2 h (Figure 3 and data not shown). This result is consistent with the predicted M.*Hha*I binding properties with ZCyt-IVA-ODN. It is also consistent with the proposal that the major role played by AdoHcy in stabilizing the flipped base in the catalytic pocket is mediated through long-range electrostatic interactions with the base and the two flanking phosphate groups (O' Gara et al., 1996).

The question remaining is the structure of ZCyt in the more slowly dissociating Phase II ZCyt-ODN: M.*Hha*I complexes and the nature of the gradual change in the ZCyt structure that allows binary complexes and ternary complexes with AdoMet to dissociate. The first clue as to the structure of ZCyt in these highly stable complexes comes from their observed electrophoretic mobility in native gels (Figure 3). All of the complexes, regardless of the presence or absence of co-factor migrate with the increased mobility that is postulated to be the result of the “closed” configuration assumed by M.*Hha*I when the active site loop (residues 80-90) locks the flipped-out target into catalytic pocket (O' Gara et al., 1996). As noted above, the same behavior was observed with complexes between M.*Hha*I and ODNs with a DZCyt target. Crystal structures of DZ13, a ternary complex of AdoHcy, M.*Hha*I and a hemimethylated substrate with a DZCyt target, showed only minimal variation from the structure of HM13, a ternary complex of AdoHcy, M.*Hha*I and a hemimethylated ODN with a Cyt target. The major differences are that

a hydrogen bond exists between the proton on N5 and a water molecule in DZ13 and that the distance between the sulfur of Cys^{81} and C6 of the target DZCyt is 3.1 Å in DZ13 vs 2.3 Å for the "partial" covalent bond distance between the same atoms in the HM13 structure, whereas a covalent bond with FCyt results in a 1.8 Å distance (See O' Gara et al., 1996 and Sheikhnejad et al., 1999 for other details). Since Cyt does not form closed complexes in the absence of co-factor, but the transition state mimic of ZCyt (DZCyt) does, our results suggest that the base in the initial Phase II complex has a similar structure to DZCyt. This would be a closed complex in which covalent bond formation has occurred leading to a non-aromatic ring structure with saturation of the 5,6 double bond between C6 and the N5 (Structure IIb). Our finding that the $t^{1/2}$ for dissociation of the more labile binary complex with ZCyt-ODN is on the order of days (Table 2) rather than hours, as was observed for non-covalent M.*Hha*I complexes formed with DZCyt-ODN (Sheikhnejad et al., 1999 and unpublished A.S.B. and J.K.C.), supports this idea. Since M.*Hha*I:ZCyt-ODN:AdoHcy complexes do not evidence significant dissociation over 24 h, it seems likely that the preservation of all of the van der Waals and electrostatic contacts between AdoHcy and ZCyt-IIb plus the additional hydrogen bond to NH_2 through water stabilize the ZCyt moiety in the transition state (IIb), preventing reversion to Structure I or ring-open forms (Structures IIIb or IVa). Modeling of AdoMet in the HM13 structure (Sheikhnejad et al., 1999) indicates that the methyl group of AdoMet greatly increases the electrostatic potential of the co-factor compared with AdoHcy, leading to the extension of AdoMet further into the DNA. The four phosphate groups of the 5'-G-(flipped C)-G-C-3' sequence lie within the contour of +1 KT/e which could lead to enhanced sequence specific binding. It is probable that the extended electrostatic interactions of AdoMet enhance ZCyt-ODN binding and account for AdoMet's ability to increase the initial rate of formation of M.*Hha*I:ZCyt-ODN:AdoMet complexes relative

to binary complexes or ternary complexes with AdoHcy. Since the majority of the ZCyt-ODN in M.*Hha*I:ZCyt-ODN:AdoMet complexes is not methylated, the measurable dissociation of these complexes (Table 2) suggests that at least some of the stabilizing effect of AdoMet on the initial complex of M.*Hha*I with ZCyt-ODN is lost once the flipped ZCyt is converted to form IIb by the addition of a proton at N5. This is consistent with the observation that AdoMet has only a small stabilizing effect on complexes formed between M.*Hha*I and DZCyt-ODN (Sheikhnejad et al., 1999). Since DZCyt has the same structure as ZCyt-IIb with the exception that a proton at C6 of DZCyt replaces the covalent bond between ZCyt-IIb and Cys^{81}, it seems likely that the slow but measurable dissociation of ZCyt-ODN from the ternary complex observed in our studies is related to the rate at which covalently linked ZCytIIb is converted to either form I or form IVa. This is also likely to be true of binary ZCyt-ODN complexes, which have the same closed conformation during native gel electrophoresis as ternary complexes with AdoMet, but dissociate almost three times faster (Table 2). These results also suggest that even though the methyl group of AdoMet impairs its ability to stabilize intermediate IIb relative to AdoHcy, it can still affect the equilibrium between form IIb and forms I or IIIb or the conversion of IIIb to IVa. However, our data do not allow us to rule out the possibility that the absence of co-factor may facilitate hydrolysis of form IIIb by allowing a more direct interaction with water molecules in the active site pocket.

SDS-PAGE as a tool for examining the determinants of stability of ZCyt-ODN:M.*Hha* I complexes. The primary criterion for identifying covalent bond formation between a C5-MTase and a cytosine analog in DNA is the ability of the DNA-enzyme complex to withstand heating at

95 ^{o}C in the presence of SDS and a reducing agent. Our studies and those of others (Gabbara & Bhagwat, 1995) clearly demonstrate that C5-MTase complexes with ZCyt-containing DNA do not withstand this treatment. We initially undertook our studies of the temperature stability of M.*Hha*I:ZCyt-ODN complexes in SDS and β-mercaptoethanol to strengthen the evidence indicating that ZCyt in these complexes is covalently linked to M.*Hha*I. The results we obtained in comparing the behavior of M.*Hha*I-FCyt- and ZCyt-ODN binary and ternary complexes by SDS-PAGE are consistent with the hypothesis that covalent bond formation occurs and provide new information about structural aspects of both types of complexes.

The first, and most surprising finding, was that both binary and ternary complexes formed by M.*Hha*I and ZCyt-ODN are not only stable to treatment with SDS and β-mercaptoethanol but that they are more compact than unbound enzyme, i.e., they have a higher electrophoretic mobility than free M.*Hha*I during SDS-PAGE. The majority of M.*Hha*I:ZCyt-ODN complexes formed during incubation at 37 ^{o}C incubation are stable even after heating at temperatures >50 ^{o}C in the presence of 1% SDS and β-mercaptoethanol. Regardless of whether co-factor is present or not, the amount of radiolabeled ZCyt-ODN that remains associated with the rapidly migrating conformer of M.*Hha*I after treatment with SDS and β-mercaptoethanol at 25 ^{o}C is approximately equivalent to that detected in the "closed" complex after native gel electrophoresis. This is a strong argument for the conclusion that the rapidly migrating enzyme-ZCyt-ODN complex is the same complex that migrates as a "closed" complex in native gels.

The behavior of M.*Hha*I:FCyt-ODN complexes subjected to SDS-PAGE is remarkably different from that of M.*Hha*I:ZCyt-ODN complexes. As we have demonstrated, while FCyt-ODN

complexes are completely stable to heating at 95 ^{o}C in the presence of 1% SDS and β–mercaptoethanol, at 22 ^{o}C, only a small percentage of the ternary complexes with FCyt-ODN migrate faster than free enzyme in SDS-PAGE gels while essentially all of ZCyt-ODN complexes do so (Figure 6). As expected, the majority of FCyt-ODN complexes migrate more slowly than unbound M.*Hha*I. However, until heated to 95 ^{o}C, only a small fraction of the population of FCyt-ODN complexes migrates as slowly as the completely denatured complex of M.*Hha*I plus a covalently bound ODN. In contrast, all M.*Hha*I:ZCyt-ODN complexes migrate at the same rate on SDS-PAGE, regardless of the presence co-factor or the temperature to which they were heated prior to loading for electrophoresis.

It has been established from X-ray crystallographic studies that the structure of the flipped, methylated, covalently linked FCyt in ternary complexes with M.*Hha*I and AdoHcy (F13) is extremely similar to DZ13 structure (Klimasauskas et al., 1994; Sheikhnejad et al., 1999). If it is assumed that ZCyt in both binary and ternary complexes of ZCyt-ODN with M.*Hha*I is covalently linked to the enzyme and has the same basic structure as DZCyt in DZ13, then the major difference between ZCyt- and FCyt-ODNs in the complexes is evident. Formation of stable complexes between M.*Hha*I and FCyt-ODNs requires transfer of a methyl group to the FCyt target while methylation of the ZCyt target in ZCyt-ODNs is not only unnecessary but is actually a rare event. Thus, our results indicate that, at 25 ^{o}C, under denaturing conditions, the covalent complex between enzyme and methylated FCyt-ODN takes on a variety of conformations ranging from tightly closed to fully “open” because the presence of the methyl group destabilizes the complex. With increased temperature, the equilibrium is shifted to the fully “open” form (Figure 6 and unpublished data, A.S.B and J.K.C.). It should be

noted that at this stage of our investigations, we can not determine which of the differently migrating forms of M.*Hha*I-FCyt-ODN-AdoHcy complex are analogous to the "open" complex in native gels. However, the finding that methylation destabilizes the interaction between a flipped cytosine and M.*Hha*I does not conflict with the X-ray crystallographic data indicating that M.*Hha*I assumes a fully closed complex with methylated covalently bound FCyt-ODN. It simply suggests that the conditions of close packing in crystals of M.*Hha*I ternary complexes favors stabilization of the closed complex. In modeling studies, O'Gara et al. (O' Gara et al., 1996) have observed that a tension develops between the methyl group on C5 of flipped MCyt and adjacent amino acids Pro^{80} and Cys^{81}. They have postulated that this tension destabilizes the enzyme-DNA complex sufficiently to lead to release of the methylated product.

Our results provide visual evidence that the "closed" conformation of methylated FCyt-ODN complexes is less stable to SDS-PAGE than the "closed" conformation of ZCyt-ODN complexes. This, in turn, suggest that SDS-PAGE gel electrophoresis provides a new tool for determining the stability of "closed" complexes between DNA methyltransferases and their flipped targets. In this regard, it is of interest that we do not find a similar range of conformations in SDS-PAGE analysis of M.*Hha*I:ZCyt-ODN complexes in comparison to M.*Hha*I:FCyt-ODN complexes. The only form detected is the fully "closed" complex that migrates more rapidly than free enzyme. With heating, the amount of this complex is decreased and the amount of free ZCyt-ODN is increased but no "open" or denatured complexes are detected. This finding supports the proposal that methylation of ZCyt residues in DNA is a relatively rare event since there is no *a priori* reason based on structure to predict that methylated ZCyt would differ greatly from MCyt in its ability to decrease the stability of the closed

M.*Hha*I:ZCyt-ODN complex. However, it leaves open the question of the nature of the process that leads to release of ZCyt-ODN from complexes heated under denaturing conditions. The data presented in Figure 8 indicate that the rate limiting step for dissociation of M.*Hha*I:ZCyt-ODN is a temperature dependent process that is not influenced by co-factor. Evidence will be presented elsewhere (ms. in preparation) demonstrating that for a variety of targets that cannot form covalent bonds with M.*Hha*I, both the structure of the target base and the presence or absence of co-factor can strongly influence the ability of "closed" M.*Hha*I: ODN complexes to withstand heating under the conditions used for the SDS-PAGE analysis reported here. Thus, the simplest explanation for the results in Figure 8 is that the starting structure is the same in binary and ternary M.*Hha*I-ZCyt-ODN complexes (ZCyt-IIb) and that the rate-limiting step for thermal dissociation of complexes is loss of the covalent link to enzyme (ZCyt-IIb) either by heat induced shift of equilibrium to ZCyt-I or ZCyt-IVa, rather than thermal denaturation of the enzyme. As discussed above, either pathway for conversion of ZCyt-IIb to a non-covalently bound form involves a change in the structure of the base that would lead to destabilization of the complex. While our results do not allow us to predict the effect of SDS, β-mercaptoethanol and heat on the balance between these two pathways, the end products of the two pathways are quite different. The one will lead to release of intact ZCyt-ODN and unmodified enzyme, whereas the other leads to release of ZCyt-IVa ODN and formylated (inactive) enzyme. The effect of temperature on the balance between these pathways has significant consequences with regard to whether a potential mutagenic lesion is left in the genome of 5-azacytidine or 5-azadeoxycytidine treated cells (Jackson-Grusby et al., 1997). Thus, it will be critical to determine whether and how much formylated enzyme is formed during the release of ZCyt-

ODNS from M.*Hha*I and mammalian DNA MTases and how the kinetics of formation of formylated enzyme are affected by changes in temperature.

In summary, using a totally defined ZCyt-ODN, we have been able to demonstrate that in complexes formed in the presence AdoMet, methylation is highly inefficient and that there is a slow but measurable release of a DNA Cytosine (C5) methyltransferase, M.*Hha*I, from covalent linkage to ZCyt. Since AdoMet concentrations in mammalian cells are generally at least four-fold higher than those of AdoHcy (Shivapurkar & Poirier, 1983), this finding suggests that understanding the detailed mechanism of binding and release of mammalian cytosine C5 methyltransferases from ternary complexes with AdoMet will be the next logical step in determining how to design better ODN inhibitors of DNA methyltransferases as anticancer drugs.

MATERIALS AND METHODS

Design and synthesis of ODNs. All ODNs were prepared using conventional automated DNA synthesis. ODNs containing 5-methylcytosine, 5-fluorocytosine (FCyt) or furanose abasic (AP) residues ("dSpacer" phosphoramidite) were prepared by the UNMC/Eppley DNA Synthesis Core Facility using commercially available phosphoramidites (Glen Research). ZCyt phosphoramidites were synthesized, incorporated into ODNs and structures were confirmed by mass spectroscopy as described by Eritja et al. (Eritja, 1997; García et al., 2001).

The sequences of the ODNs used in these studies are listed in Table 1. For simplicity, the following abbreviations are used in the text to refer to the ds ODNs formed by annealing these ODN pairs:

ds ODNs Designation	ss ODNs annealed
ZCyt-ODN	BZ_7 and $B'M_6$ or BZ_7 (24) and B'M (24)
Cyt-ODN	B (24) and B'M (24)
AP-ODN	BX_7 and $B'M_6$
FCyt-ODN	AFp and A'Mp

Double stranded 13-bp ODNs were formed by annealing the ss ODNs at 37 °C for 60 minutes. BZ_7 (24) and B'M (24) were annealed by heating at 65 °C for 10 minutes and slowly cooling to ~45 °C over a 60 minute period. All other ODNs were annealed by heating to 90 °C for 10 minutes and slowly cooling to ~45 °C over a 60 minute period.

Assays of inhibitor methylation and inhibitory potency

Analysis of methyltransferase inhibitors - To determine the effect of inhibitory ODNs on the rate of substrate methylation, sets of duplicate reactions (50 μL) in Methylation Reaction (MR) buffer [50 mM Tris (pH 7.5), 10 mM EDTA, and 5 mM β-mercaptoethanol] containing 8.6 nM M.*Hha*I, 2.43 μM ^{3}H-AdoMet (Specific activity = 15 Ci/mmol, 1 Ci = 37 GBq) and increasing concentrations of substrate AM_p:A' were incubated at 37°C for 5 minutes in the absence or presence of FCyt-ODNs (30 nM, 60 nM, or 120 nM) or ZCyt-ODNs (15 nM, 30 nM, and 60 nM). Samples were processed for determining the amount of $[^3H]CH_3$ incorporated as described previously (Creusot et al., 1982; Sheikhnejad et al., 1999). Briefly, NaOH was added

to a final concentration of 0.3 M to terminate the reaction, followed by the addition of salmon sperm carrier DNA to a final concentration of 70 μg/mL and perchloric acid (PCA) to a final concentration of 0.86 M. Precipitated DNA was collected and washed on glass fiber filters prior to analysis for ^{3}H content by liquid scintillation counting in aqueous fluor (ScintiSafe Plus 50%, Fisher). Lineweaver-Burk plots of the resulting data were prepared to determine the K_i values and the type of inhibition exerted by FCyt and ZCyt-ODNs. Highly purified M.*Hha*I (Kumar et al., 1992) used for all experiments was the generous gift of Dr. S. Kumar, New England Biolabs, Inc. or Dr. X. Cheng, Emory University.

Methylation of ZCyt- or FCyt-ODNs - Sets of duplicate reaction mixtures (25 μL) in MR buffer contained 173 nM M.*Hha*I, 8 μM [^{3}H]CH_3-AdoMet (Specific activity = 84 Ci/mmol), and the indicated concentrations of ZCyt-ODN (BZ_7:B'M_6) or FCyt-ODN over a range of 50 – 400 nM. These were incubated at 37°C for 30 minutes and [^{3}H]CH_3 incorporation measured as above. Plots of data were constructed using GraphPad Prism 3.0 software.

Analysis of binary and ternary M.*Hha* I-ODN complexes

Native gel shift assays - Binding reaction mixtures containing 45 nM ^{32}P-endlabeled ZCyt-ODN, 108 nM M.*Hha*I, 75 ng poly(dAdT:dAdT), and 100 μM co-factor (as indicated in figure) in M.*Hha*I binding buffer (50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol, and 13% (v/v) glycerol) were incubated at 22°C for 30 minutes as previously described (Sheikhnejad et al., 1999). One hundred-fold excess unlabeled (4.5μM) ZCyt-ODN was added to each binding reaction following the initial incubation and incubations continued at 22°C for the indicated times. Complexes were analyzed by electrophoresis at 150 V for 2.5-3

hrs on 10 % native polyacrylamide gels that had been pre-run at 100 V for 1 hour in TBE buffer (89 mM Tris borate, pH 8.0; 2 mM EDTA).

Dried gels were autoradiographed by exposure to Dupont Cronex 4 medical X-ray film for 1-24 hours. Band intensity was quantified by PhosphorImager (Molecular Dynamics) analysis. The percentage of ODN in complexes was determined for each lane by dividing the signal in the band containing complex by the sum of the signals from bands containing free ODN and ODN:enzyme complex and multiplying by 100. For dissociation analysis, the natural log of the percentage of ODN in complexes for each lane was calculated and plotted versus the time of competition. The (-) slope of these plots is equal to the k_{off}, and $T_{1/2}$ (= $0.693/k_{off}$) was calculated.

SDS-PAGE binding assays - To assess the stability of complexes under denaturing conditions, radiolabeled ds ODN, M.*Hha*I, and co-factor (concentrations indicated in captions) in M.*Hha*I binding buffer were incubated at 37 °C. After 60 minutes, reaction mixtures were brought to final concentrations of 10% (v/v) glycerol, 1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol, heated at indicated temperatures for 5 minutes, loaded directly onto 10% SDS-polyacrylamide gels, and electrophoresed at 200 V for 60 minutes. Dried gels were subjected to autoradiographic analysis as described above. To obtain the results presented in Figure 5, the gel was rehydrated following autoradiographic analysis in a solution containing 3% (v/v) glycerol, 30% (v/v) methanol and 10% (v/v) acetic acid and was stained with Coomassie blue (GELCODE-Blue reagent, Pierce) according to the manufacturers instructions.

Urea-PAGE Analysis of ODNs released from M.HhaI complexes - Binding reactions containing radiolabeled ZCyt-ODN, M.*Hha*I, and co-factor in M.*Hha*I binding buffer were incubated at 37°C. After 60 minutes, samples were brought to a final concentration of 1% SDS and β-mercaptoethanol. Tracking dye was added to samples to final concentrations of 0.25% xylene cyanole, 0.25% bromophenol blue, and 30% glycerol. After heating at 50 °C or 95 °C for 5 minutes, samples were loaded on a 15% acrylamide/8M urea gel, and electrophoresed at 200 V for 30 minutes, the time required for the dye front to reach the bottom of the gel. Dried gels were subjected to autoradiographic analysis as described above.

ACKNOWLEDGEMENTS

Partial support for this work was provided by the DAMD Breast Cancer Program (DAMD 17-98-1-8215) to JKC and fellowship support from the Graduate College at UNMC to ASB. We are also greatful to Drs. X. Cheng and S. Kumar for their generous gifts of purified M.*Hha*I.

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FIGURE CAPTIONS

Figure 1. Inhibition of M.*Hha*I by FCyt- or ZCyt-ODNs. Methylation reactions containing 8.6 nM M.*Hha*I, 2.4 μM [^{3}H]CH_3-AdoMet, and increasing concentrations (40, 80, 160, 320 and 640 nM) of AMp:A' substrate (Table 1) were incubated (A) without inhibitor (λ) or with 30 nM (υ), 60 nM (ν), or 120 nM (σ) FCyt-ODN. In (B), the same concentrations of enzyme, cofactor, and substrate were incubated without inhibitor (λ) or with 15 nM (υ), 30 nM (ν), or 60 nM (σ) of ZCyt-ODN (BZ_7:B'M_6). AMp:A' contains a single, hemi-methylated M.*Hha*I recognition site and target cytosine. For both (A) and (B), the rate of incorporation of [^{3}H]CH_3 groups was determined as described in Experimental Procedures, and the inverse of the rate of substrate methylation was plotted versus the inverse concentration of substrate.

Figure 2. Effect of co-factor on M.*Hha*I: ZCyt-ODN complex dissociation. Binding reactions containing 45 nM ^{32}P-end-labeled ZCyt-ODN (BZ_7:B'M_6, with all radiolabel at the 5' end of the B'M_6 strand), 108 nM M.*Hha*I, 75 ng poly(dAdT:dAdT) and either no co-factor (lanes 1-5), 100 μM AdoMet (lanes 6-10), or 100 μM AdoHcy (lanes 11-15) were incubated at 22°C for 30 minutes. One hundred-fold excess (4.5μM) of unlabeled ZCyt-ODN (BZ_7:B'M_6) was added to each binding reaction (except for those loaded in lanes 1,6, and 11, indicated by minus sign) following the initial incubation period. Incubations were continued for the times indicated. In lanes 2,7, and 12 where "0 hours" is indicated for the time of competition, one hundred-fold excess of unlabeled ZCyt-ODN was added to the binding reaction mixture immediately before loading on a native polyacrylamide gel. Values for "% Bound" were derived from PhosphorImager analysis of the dried gel (% Bound = [Counts in complex band/ (Counts in complex band + Counts in free ODN band)] x 100).

Figure 3. Analysis of M.*Hha*I: ^{32}P-ZCyt-ODN complex dissociation. From native complex dissociation experiments (e.g., Figure 2), the percentage of total ODN associated with M.*Hha*I was quantified and plotted versus the duration of competition of M.*Hha*I: ^{32}P-ZCyt-ODN (BZ_7:B'M_6) complex with homologous unlabeled ZCyt-ODN. The presence of AdoHcy (σ), AdoMet (ν) or no co-factor (υ) during M.*Hha*I:^{32}P-ZCyt-ODN complex formation and dissociation is indicated. "Phase I" represents the initial two hours of competition in which the dissociation of M.*Hha*I:^{32}P-ZCyt-ODN complexes was at least 100 fold more rapid than in "Phase II" (2-24 hours competition).

Figure 4. Effect of heating on the integrity of ZCyt-ODNs before and after formation of complexes with M.*Hha*I. Reactions containing 0.256 μM ^{32}P-5'-endlabeled ZCyt-ODN (^{32}P-BZ_7-24:B'M-24), 0.216 μM M.*Hha*I (lanes 3-6) and 0.16 mM AdoMet (lanes 3-4) or AdoHcy (lanes 5-6) were incubated at 37°C for 60 minutes. Following the addition of 1% SDS and β-mercaptoethanol, the samples were heated at 95°C (+, lanes 2,4,6) or 50°C (lanes 1,3,5) for 5 minutes. Samples were analyzed on a 15% acrylamide/8 M urea gel. The arrows indicate the migration of ss 24-mer ODNs and 13-mer ODNs used as markers.

Figure 5. Analysis of M.*Hha*I complexes with FCyt- and ZCyt-ODNs under denaturing conditions. Reactions containing either no ODN (lanes 1-2) or 2.96 μM radiolabeled FCyt- (lanes 3 and 4) or ZCyt-ODN (lanes 5-7), 1.35 μM M.*Hha*I, and 200 μM AdoMet (omitted from reactions in lanes 1 and 2) were incubated for 1 h at 37^{o}C. Following complex formation, 1% SDS and β-mercaptoethanol were added. The samples were either held at room temperature

(lanes 2, 4, 7) or heated for 5 minutes at 95°C (lanes 1, 3, 5) or 75°C (lane 6), loaded unto a 10% SDS-polyacrylamide gel, and electrophoresed at 200 V for ~60 minutes. The resulting gel was: stained with Coomassie blue and (B) examined by autoradiographic imaging. The molecular weight markers are indicated to the left of images in both (A) and (B). FCyt-ODN is AFp:A'Mp with ^{32}P 5'-labeled A'Mp and ZCyt-ODN is BZ_7:$B'M_6$ with ^{32}P 5'-end labeled $B'M_6$.

Figure 6. Analysis of the effect of co-factor on stability of M.*Hha*I:ZCyt-ODN complexes under denaturing conditions. Reactions containing 41nM ^{32}P-ZCyt-ODN (BZ_7:$B'M_6$, 5'-endlabel on $B'M_6$), 108nM M.*Hha*I, and 100 μM co-factor (as indicated) were incubated at 37°C for 60 minutes. Reactions were terminated by bringing them to a final concentration of 10% (v/v) glycerol, 1% (v/v) SDS, and 1% (v/v) 2-mercaptoethanol. Products were either left unheated or heated at indicated temperatures for 5 minutes prior to loading and electrophoresed as described in Figure 5. An autoradiographic image is shown with molecular weight markers indicated to the right.

Figure 7. The effect of increasing incubation temperatures prior to SDS-PAGE analysis of M.*Hha*I:ZCyt-ODN complexes. To determine the differences in complex stability under denaturing conditions that depended on co-factor, M.*Hha*I:^{32}P-ZCyt-ODN complexes formed in the absence of co-factor (υ), or presence of excess AdoMet (ν) or AdoHcy (σ) were either left unheated or heated at 50 or 75°C prior to SDS-PAGE . All details are as described in Figure 6. The total percentage of SDS resistant complex present in unheated reactions after SDS-PAGE was set as 100%. The percentage of complex remaining was plotted versus the increasing temperature of heating prior to loading.

Figure 8. Comparison of FCyt- and ZCyt-ODN methylation by M.*Hha*I. Reaction mixtures containing 173 nM M.*Hha*I, 450 nM [^{3}H]CH_3-AdoMet (specific activity = 84 Ci/mmol), and increasing concentrations (50 nM, 100 nM, 200 nM, or 400 nM) of ZCyt-ODNs (ν) or FCyt-ODNs (υ) were incubated at 37°C for 30 minutes. Reaction products were precipitated in perchloric acid as previously described (Sheikhnejad et al., 1999) and the amount of methyl transfer to the ODNs was determined by liquid scintillation counting.

Figure 9. Predicted structural changes in ZCyt in DNA. (I) Intact ZCyt; (IIa) Dihydrocytosine intermediate form of ZCyt following addition of water across the C6-N5 double bond; (IIb) Dihydrocytosine intermediate form of ZCyt following enzymatic attack at C6; Ring open form of ZCyt in the absence (IIIa) or presence (IIIb) of DNA C5-MTase. Following hydrolysis of either ring open form of ZCyt, the product would be form (IVa) which could take on the conformation shown as form (V). Theoretically (V) in DNA can direct incorporation of Cyt during DNA replication leading to C:G→ G:C transversions (Jackson-Grusby et al., 1997).

Table 1. Primary sequence of ss ODNs used in this study.

ss ODN Designation	Nucleotide Sequence
BZ_7	5’ TGT CA**G** **<u>Z</u>GC** ATG G 3’
BX_7	5’ TGT CA**G** **<u>X</u>GC** ATG G 3’
$B'M_6$	5’ CCA T**GM GC**T GAC A 3’
BZ_7-24	5’ TGT CA**G** **<u>Z</u>GC** ATG GAT GGT TAT AAT 3’
B-24	5’ TGT CA**G** **<u>C</u>GC** ATG GAT GGT TAT AAT 3’
B’M-24	5’ ATT ATA ACC ATC CAT **GMG C**TG ACA 3’
A	5’ ATT **G<u>C</u>G C**AT TCC GGA TCC GCG ATC 3’
AMp	5’ ATT **GMG C**AT TCM GGA TCM GMG ATC 3’
A’Mp	5’ GAT MGM GGA TCM GGA AT**G MGC** AAT 3’
$A'M_{19}$	5’ GAT CGC GGA TCC GGA AT**G MGC** AAT 3’
A’	5’ GAT CGC GGA TCC GGA AT**G <u>C</u>GC** AAT 3’
AFp	5’ ATT **G<u>F</u>G C**AT TCF GGA TCF GFG ATC 3’

Sequence abbreviations: Z = ZCyt, F = FCyt, X = abasic furanose, M = 5-methyl cytosine. Annealing of ss target ODNs (designated A or B) to complementary methylated ODNs (designated A’ or B’) leads to the formation of ds ODNs that contain a single target base (underlined) in a hemi-methylated M.*Hha*I recognition site (boldface). The B:B’ 13-bp ds ODNs have the same base sequence (with the exception of the target base) as the ODNs used in M.*Hha*I crystal structure studies (Klimasauskas et al., 1994; O' Gara et al., 1996; O' Gara et al., 1995). All ODNs were analyzed by polyacrylamide gel electrophoresis to confirm that all elongation cycles had been completed.

Table 2. Summary of M.*Hha*I:ODN complex dissociation experiments.

	ZCyt-ODN				AP-ODN
	Phase I		**Phase II**		**-**
Co-factor Present	**k_{off} (hrs^{-1})**	**$t_{1/2}$ (hrs)**	**k_{off} (hrs^{-1})**	**$t_{1/2}$ (hrs)**	**$t_{1/2}$ (hrs)**
None	0.878	1.1	0.0093	110	90
AdoHcy	0.724	1.4	0.0009	1100	260
AdoMet	0.899	1.1	0.0032	310	180

The natural log of the percentage of total ODN associated with M.*Hha*I during native complex dissociation analysis (Figures 3 and 4) was calculated and plotted versus the duration of competition of pre-formed complexes containing radiolabeled ODNs with homologous, unlabeled ODNs. k_{off} (= (-)(slope) of the plot), and the $t_{1/2}$ (= 0.693/k_{off}) were determined from these plots. The dissociation of M.*Hha*I complexes containing ZCyt-ODN in the absence of co-factor, or presence of AdoHcy or AdoMet is presented as two sets of resulting k_{off} and $t_{1/2}$ values for "Phase I" (0-2 hrs competition) and "Phase II" (2-24 hrs competition) because of the observed biphasic dissociation of the complexes (Figure 4). Dissociation of AP-ODN in the presence or absence of cofactors is presented as a single set of k_{off} and $t_{1/2}$ values since the dissociation plot was linear.

ABBREVIATIONS

Ade, Adenine; AdoHcy, *S*-Adenosyl homocysteine; AdoMet, *S*-Adenosyl methionine; AP, abasic site; DNA C5-MTase, DNA (Cytosine-C5)-methyltransferase; Cyt, Cytosine; ds, double-stranded; DZCyt, 5,6-dihydro-5-azacytosine; FCyt, 5-fluorocytosine; MCyt, 5-methyl cytosine; M.*Eco*RII, *Eco*RII methyltransferase; M.*Hha*I, *Hha*I methyltransferase; M.*Hpa*II, *Hpa*II methyltransferase; MR Buffer, Methylation Reaction Buffer; ODN, Oligodeoxyribonucleotide; ss, Single-stranded; TE, Tris-EDTA; Ura, Uracil; ZCyt, 5-azacytosine; ZCyd, 5-azacytidine; ZdCyd, 5-azadeoxycytidine

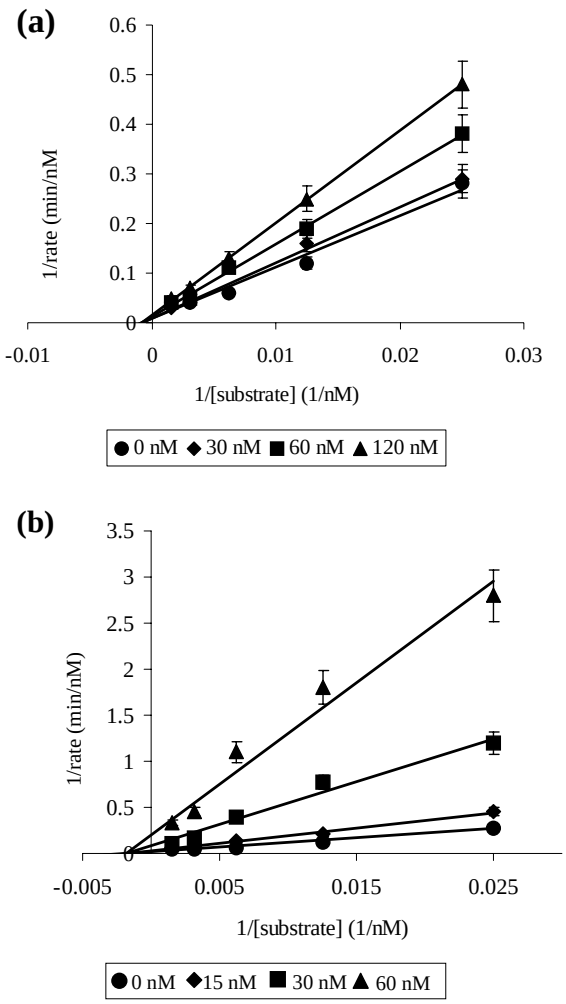
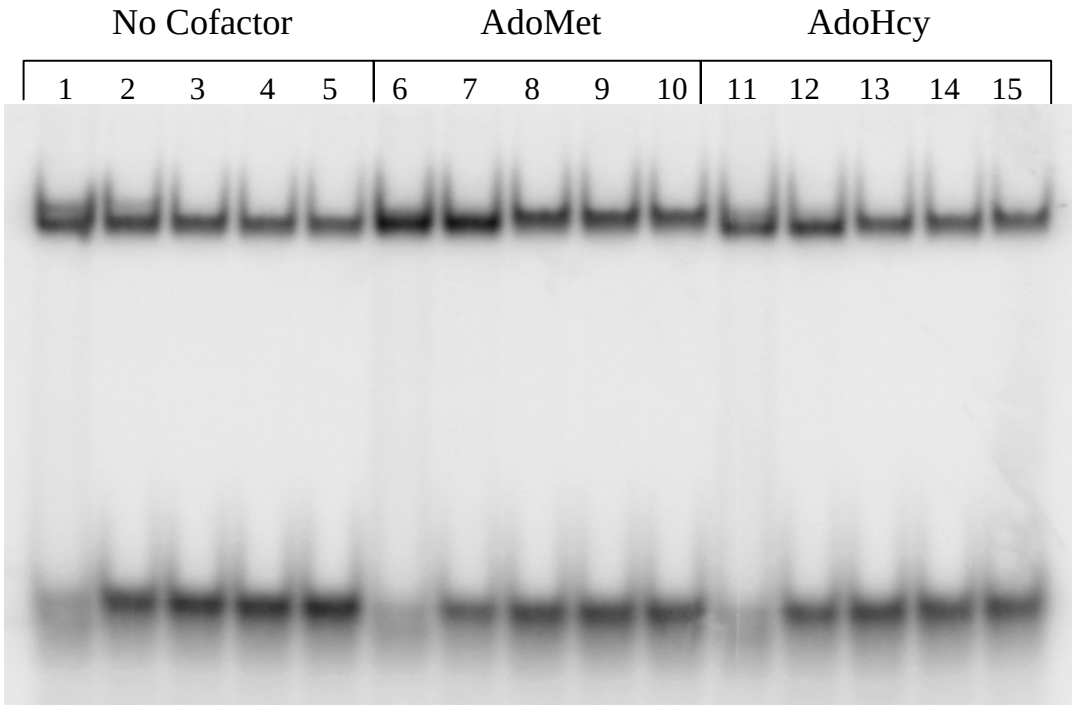

(a)
0.6
0.5
0.4
0.3
0.2
0.1
0
1/rate (min/nM
-0.01
0
0.01
0.02
0.03
1/[substrate] (1/nM)
0 nM
30 nM
60 nM
120 nM
(b)
3.5
3
2.5
2
1.5
1
0.5
0
1/rate (min/nM)
-0.005
0.005
0.015
0.025
1/[substrate] (1/nM)
0 nM
15 nM
30 nM
60 nM

Figure 1



% Bound	89	57	48	43	38	95	74	62	58	55	89	58	46	48	49
Dissoc. Time (h)	-	0	6	10	24	-	0	6	10	24	-	0	6	10	24

Figure 2

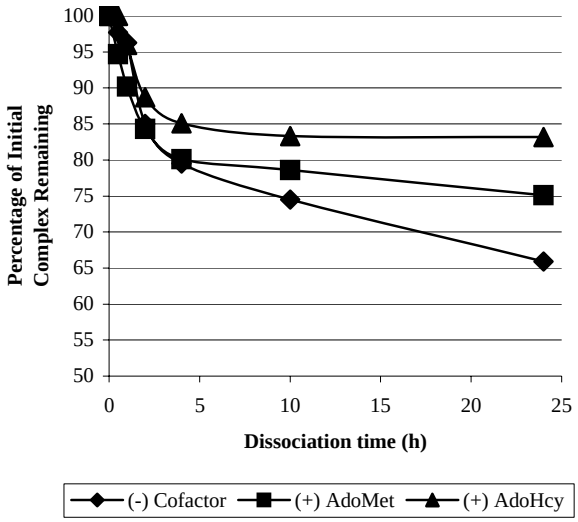

Percentage of Initial Complex Remaining
100
95
90
85
80
75
70
65
60
55
50
0
5
10
15
20
25
Dissociation time (h)
(-) Cofactor
(+) AdoMet
(+) AdoHcy

Figure 3

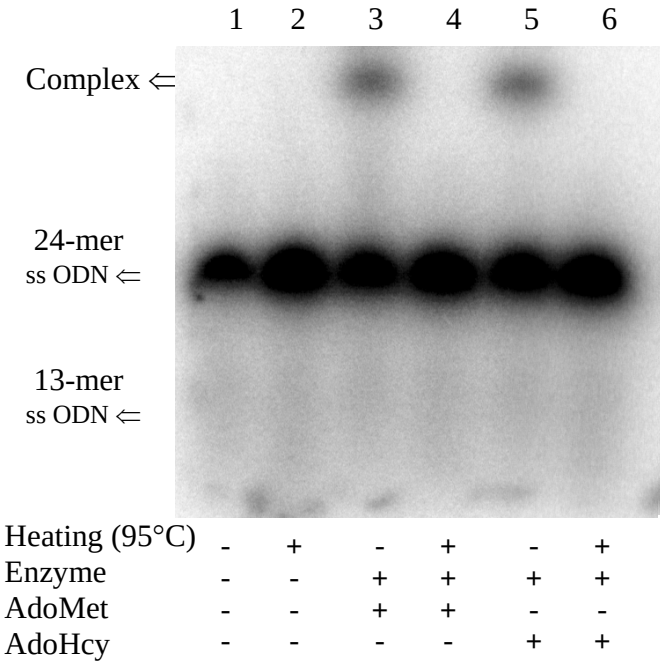

1 2 3 4 5 6
Complex ⇐
24-mer
ss ODN ⇐
13-mer
ss ODN ⇐
Heating (95°C) - + - + - +
Enzyme - - + + + +
AdoMet - - + + - -
AdoHcy - - - - + +

Figure 4

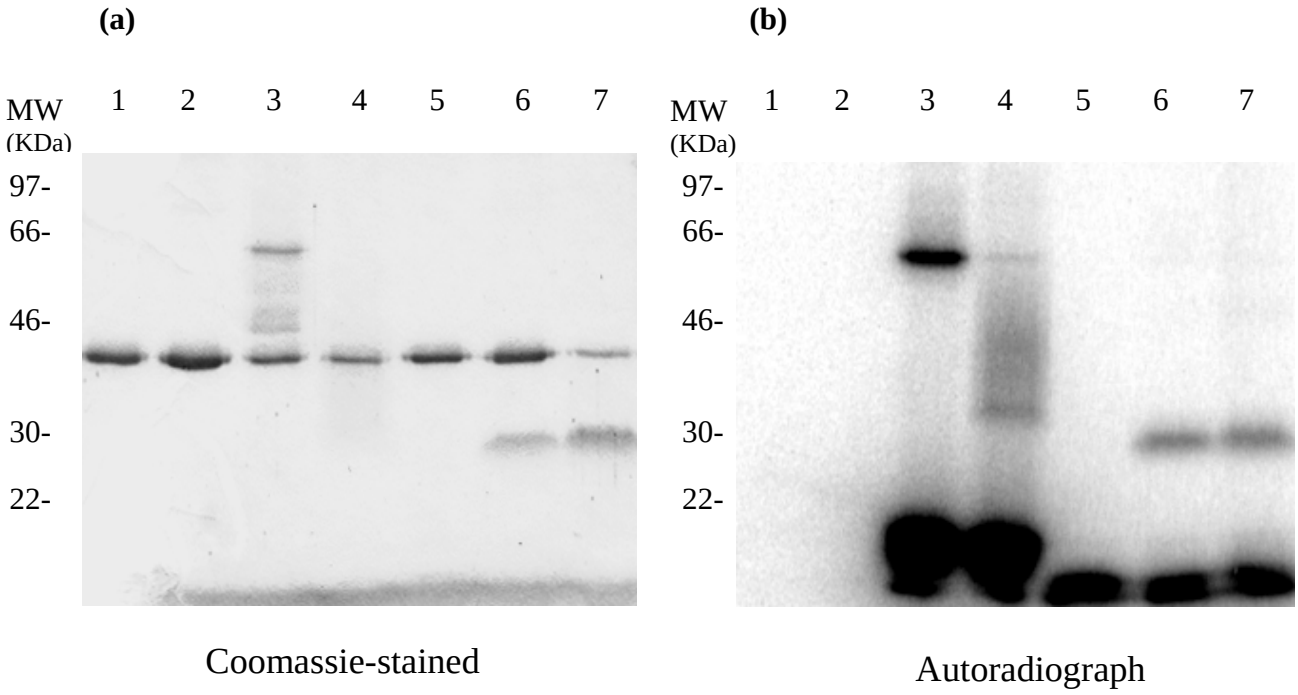

(a)
(b)
MW
(KDa)
1 2 3 4 5 6 7
97-
66-
46-
30-
22-
MW
(KDa)
1 2 3 4 5 6 7
97-
66-
46-
30-
22-
Coomassie-stained
Autoradiograph

Figure 5

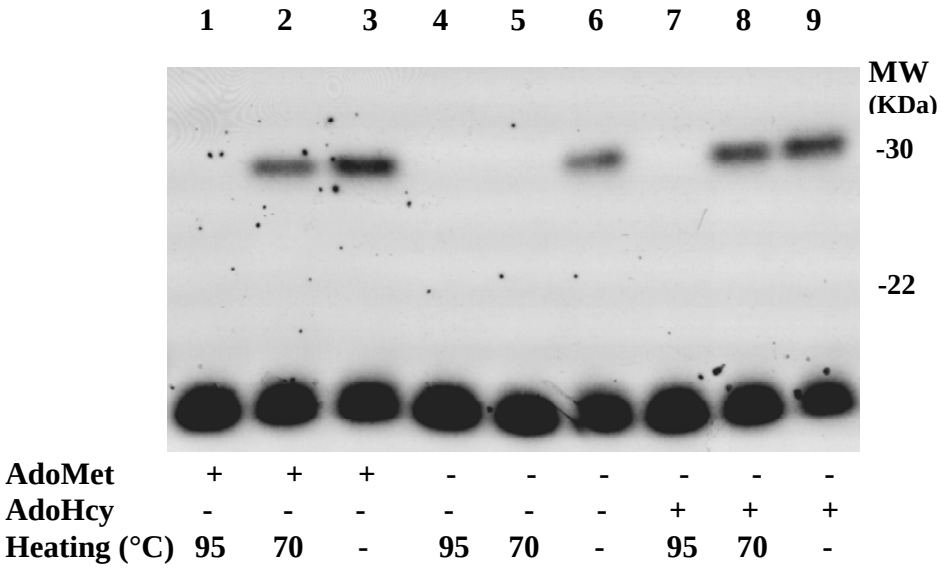

1 2 3 4 5 6 7 8 9
MW
(KDa)
-30
-22
AdoMet + + + - - - - - -
AdoHcy - - - - - - + + +
Heating (°C) 95 70 - 95 70 - 95 70 -

Figure 6

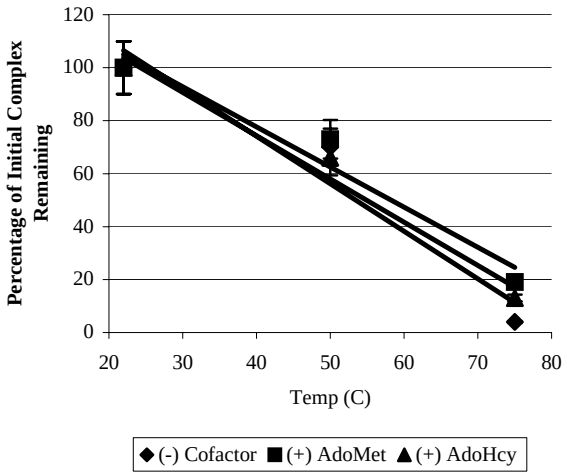

Percentage of Initial Complex Remaining
120
100
80
60
40
20
0
20
30
40
50
60
70
80
Temp (C)
(-) Cofactor
(+) AdoMet
(+) AdoHcy

Figure 7

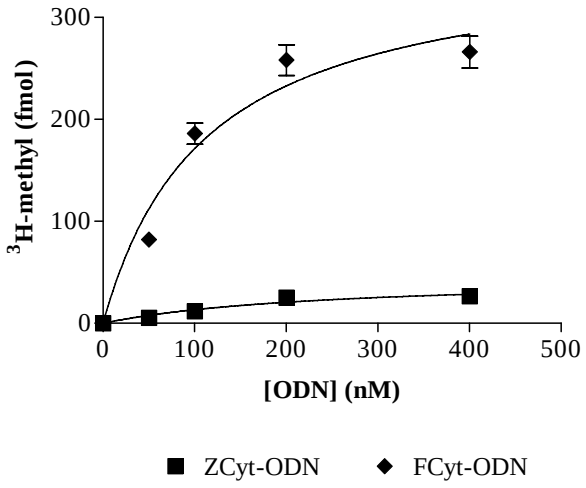

300
200
100
0
0
100
200
300
400
500
^{3}H-methyl (fmol)
[ODN] (nM)
ZCyt-ODN
FCyt-ODN

Figure 8

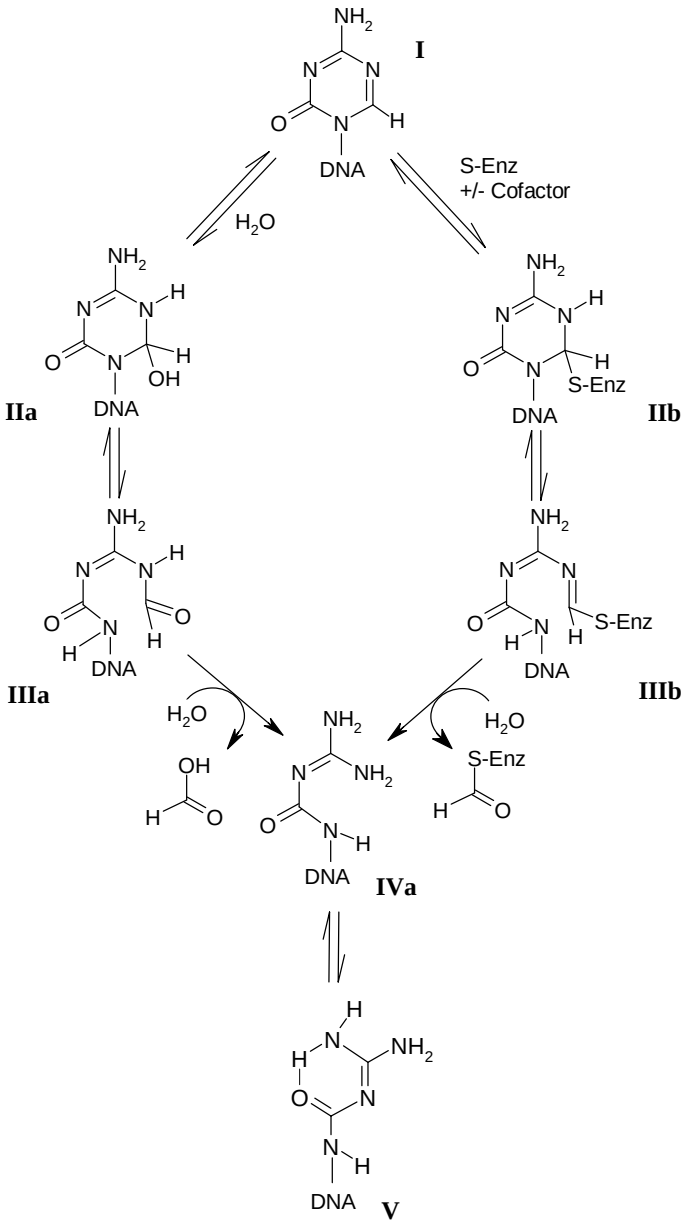


Figure 9