

The mechanism of inhibition of DNA (cytosine-5-)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor

Sam GABBARA* and Ashok S. BHAGWAT†

Department of Chemistry, Wayne State University, Detroit, MI 48202, U.S.A.

The mechanism of inhibition of DNA (cytosine-5-)-methyltransferases by the mechanism-based inhibitor 5-azacytosine has remained unclear, mainly because of the unavailability of a substrate in which the inhibitor, but not normal cytosine, is present at the target site. We synthesized an oligonucleotide duplex containing a single target site for the *EcoRII* methyltransferase, in which the target base is 5-azacytosine. This substrate formed a stable covalent complex with *EcoRII* methyltransferase in the absence and in the presence of the cofactor *S*-

adenosylmethionine. The complex formed in the presence of the cofactor was resistant to SDS and moderate heat treatment, and a methyl group was incorporated into the complex. Enzyme titration and kinetic studies of inhibition suggest that methyl transfer to the complex occurred only during the first turnover of the reaction. These results suggest that, when the enzyme binds to 5-azacytosine in the presence of the cofactor, a methyl group is transferred to the N-5 position of the base, resulting in the inactivation of the enzyme.

INTRODUCTION

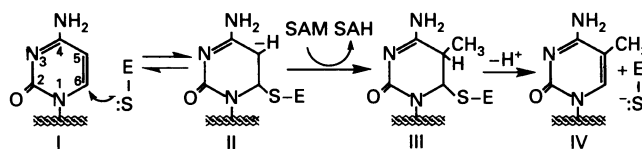
DNA (cytosine-5-)-methyltransferases (C5 MTases) catalyse the transfer of a methyl group from *S*-adenosylmethionine (SAM) to position 5 of cytosine in specific DNA sequences. As a result of the methyl-group transfer, SAM is converted into *S*-adenosylhomocysteine (SAH). The methylation reaction is thought to occur by an addition/elimination mechanism [1; Scheme 1).

5-Azacytosine-containing DNA (azaC DNA) has been shown to inhibit the activity of the bacterial C5 MTases, *M.EcoRII* [2,3], *M.HpaII* [3,4] and *M.MspI* [3]. It has also been shown to inhibit the activity of a mammalian MTase *in vitro* [5]. These enzymes form stable covalent complexes with such DNA. Santi et al. [1] proposed a mechanism for the inhibition of C5 MTases by 5-azacytosine that occurs in a SAM-independent fashion. It involves a nucleophilic attack by the enzyme at the reactive C-6 position of cytosine, followed by proton transfer to N-5 of the pyrimidine ring (Scheme 2). This mechanism of inhibition provides an explanation for the hypomethylation of DNA that has been observed when cell lines are treated with 5-azacytidine [6–8]. The drug is taken up by the cell, converted into 5-azadeoxycytidine triphosphate and then incorporated into DNA to act as a mechanism-based inhibitor of C5 MTases.

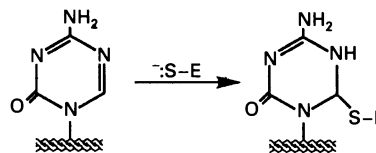
For another mechanism-based inhibitor, 5-fluorocytosine, methyl transfer to the inhibitor has been demonstrated. DNA containing 5-fluorocytosine at the site of methylation has been shown to form irreversible covalent complexes with several bacterial C5 MTases [9–13] and one eukaryotic enzyme [14]. For two of these enzymes, the covalent complex was shown to contain methyl groups [10,11].

Although Scheme 2 explains how azaC may inhibit C5 MTases without the transfer of methyl groups to DNA, there is no chemical reason why the enzymes should not transfer methyl

groups to 5-azacytosine in DNA. Transfer of a methyl group to 5-azacytosine was not tested in any of the previous studies, because in those studies azaC DNA was prepared from bacterial cultures treated with 5-azacytidine [3,4,15]. Such preparations resulted in DNA molecules with the drug incorporated at sites for methyl transfer as well as at sites that were not targets for methyl transfer. Additionally, as the incorporation of 5-



Scheme 1 Mechanism of cytosine methylation



Scheme 2 The SAM-independent mechanism of the inhibition of C5 MTase by 5-azacytosine

∴ S-E denotes C5 MTase. This mechanism was proposed by Santi et al. [1].

Abbreviations used: C5 MTase, DNA (cytosine-5-)-methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; azaC DNA, 5-azacytosine-containing DNA; 5-azadCTP, 5-azadeoxycytidine triphosphate.

* Present address: Department of Biological Chemistry, University of Michigan; and Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105, U.S.A.

† To whom correspondence should be addressed.

azacytosine in DNA was inefficient, a majority of the sites of methylation contained normal cytosine instead of 5-azacytosine. To avoid these problems, we have enzymically synthesized a DNA substrate in which 5-azacytosine was incorporated at a single site which is a target for the *Escherichia coli* methyltransferase *EcoRII* (*M.EcoRII*). Using this system, we have re-examined the role of SAM in the formation of adducts between C5 MTases and 5-azacytosine in DNA. Our results modify the mechanism proposed by Santi et al. [1], and suggest that the enzyme transfers a methyl group to the N-5 position in 5-azacytosine.

MATERIALS AND METHODS

Formation of oligonucleotide duplexes containing single *EcoRII* sites

The oligonucleotides that make up the template–primer hybrid (duplex I) were synthesized at the Macromolecular Facility, Wayne State University School of Medicine, and were purified by using NENSORB PREP cartridges (Du Pont–New England Nuclear) according to the manufacturer's recommendations. 5-Azadeoxycytidine triphosphate (5-azadCTP) was synthesized as previously described [16], and was generously given by Dr. S. Friedman (SUNY Health Sciences Center at Brooklyn). The template strand (25-mer, Table 1) was mixed with the primer strand (15-mer, Table 1) in TE buffer (10 mM Tris/HCl, pH 7.8, 1 mM EDTA, pH 7.8), and the mixture was heated at 90 °C for 3 min and then slowly cooled to 25 °C. Duplexes II and III were synthesized by the extension of the primer in duplex I. The polymerization reaction contained 1–3 μ M of duplex I, 20–30 μ M dATP, dTTP and dGTP (Pharmacia–LKB), either 30 μ M dCTP (Pharmacia–LKB) or 5–20 μ M 5-azadCTP, and 0.3 unit/ μ l Sequenase v. 1.0 (United States Biochemical) in polymerization buffer (40 mM Tris/HCl, pH 7.5, 20 mM MgCl₂, 50 mM NaCl, 10 mM dithiothreitol, 5 mM MnCl₂). Reactions were carried out at 25 °C for 5–15 min. After polymerization, EDTA was added to a final concentration of 10 mM, and the reaction mixture was extracted once with phenol/chloroform, pH 7.6, and once with chloroform, and then desalted by spin-dialysis through a Sephadex G-50 column (Pharmacia–LKB). For *Bst*NI digestion of duplexes, duplexes II or III were end-labelled with ³²P in both strands and then incubated with 10 units of *Bst*NI endonuclease (New England Biolabs) in a 20 μ l volume of 10 mM Tris/HCl (pH 7.8)/150 mM NaCl/10 mM MgCl₂/10 mM 2-mercapto-

ethanol. These reactions were performed at 37 °C for 1 h. The reaction products were separated by polyacrylamide-gel electrophoresis with 10% polyacrylamide in TBE (90 mM Tris/90 mM boric acid/2 mM EDTA, final pH 8.1) electrophoresis buffer, and were revealed by autoradiography using Fuji X-ray film RX. To quantify the amount of primer–template hybrid that was converted into duplex II or III, the autoradiographs were scanned with the VISAGE electrophoresis-gel analysis system (Millipore Corp.).

Purification of *M.EcoRII*

The MTase was purified by four chromatography steps. Purification of the MTase on a cellulose phosphate column has been described previously [12]. After this step, the enzyme was purified to apparent homogeneity by successive chromatography on DEAE-Sephacel, FPLC Superdex 75 and heparin–Sephacel columns (S. Gabbara and A. Bhagwat, unpublished work).

DNA-binding reactions and detection of DNA–enzyme complexes

All reactions were carried out in 1 \times methylase buffer (100 mM Tris/HCl, pH 7.8, 20 mM EDTA, pH 8.0, 0.4 mM dithiothreitol) at 37 °C. Both strands of the DNA duplexes were labelled at the 5' end with ³²P as previously described [17]. Each reaction contained 0.1 pmol of duplex II and 8.5 pmol of enzyme in a final volume of 20 μ l, and it was incubated at 37 °C for 1 h. When SAM or SAH was included in the reaction, it was present at 100 μ M. The reaction was terminated by adding SDS to a final concentration of 0.8%. In some cases the reaction mixture was heated at 70 °C for 5 min. Electrophoresis was carried out at 25 °C in 10% polyacrylamide gels in TBE electrophoresis buffer at 150 V for 4 h; 0.1% SDS was included in both the gel and the electrophoresis buffer. Autoradiographs of the gels were prepared by using Fuji X-ray film RX.

Measurement of methyl-group transfer to normal and azaC DNA

Transfer of methyl groups from *S*-adenosyl-L-[methyl-³H]methionine ([*Me*-³H]SAM) (NEN–Dupont) to duplex II and III was quantified. In these experiments 8.5 pmol of enzyme was incubated with 0.18 pmol of unlabelled duplex and 15 pmol of [*Me*-³H]SAM (sp. radioactivity 72.5 Ci/mmol) in 20 μ l of 1 \times methylase buffer. Reactions were performed at 37 °C for 1 h. When the reaction was complete, the enzyme was denatured by adding SDS to final concentration of 0.5%, followed by heating at 70 °C for 5 min. Excess [*Me*-³H]SAM was removed by spin-dialysis through a Sephadex G-50 column. Samples were loaded on a polyacrylamide gel and electrophoresed under denaturing conditions as described above. In parallel experiments, a reaction between the enzyme (8.5 pmol), ³²P-labelled duplex II (0.6 pmol) and 1 nmol of unlabelled SAM in 20 μ l of 1 \times methylase buffer was performed under the same conditions, and the reaction was terminated as above. The resulting products were electrophoresed in the same gel, and the bands from the ³²P-labelled DNA were used to locate the ³H-labelled DNA. Autoradiography was carried out, the DNA was eluted from gel slices by soaking the slices overnight in 0.1% SDS/2.5 mM EDTA, and the radioactivity was quantitated by scintillation counting.

The time-dependent methyltransferase assays were performed by incubating *M.EcoRII* (0.17 pmol) with duplex II (0.17 pmol), duplex III (0.24 pmol) or duplex I (1.9 pmol) and 16 nmol of [*Me*-³H]SAM (sp. radioactivity 72.5 Ci/mmol) in a 20 μ l volume of 1 \times methylase buffer at 37 °C for the indicated time periods. Reactions were terminated by adding SDS to final concentration

Table 1 Sequences of the DNA duplexes

The *EcoRII* site is underlined for emphasis. 'me' represents the methylation at position 5 of cytosine. The target cytosine is in **bold**. Abbreviation: X, 5-azacytosine.

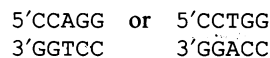
Duplex	Sequence
I	<pre> me 5'CTTATCCCAGGAATCTGTCGCAGAC 3'CTTAGACAGCGTCTG </pre>
II	<pre> me 5'CTTATCCCAGGAATCTGTCGCAGAC 3'GAATAGGGTXCTTAGACAGCGTCTG </pre>
III	<pre> me 5'CTTATCCCAGGAATCTGTCGCAGAC 3'GAATAGGGTCCCTTAGACAGCGTCTG </pre>

of 0.5% and heating at 70 °C for 5 min, and the proteins in the reactions were digested with proteinase K (final concn. 0.1 mg/ml) at 50 °C for 1 h. Sephadex G-50 columns were used to remove unincorporated [*Me*-³H]SAM. Radioactivity was quantified directly without separating the reaction products in polyacrylamide gels.

The enzyme-titration methyltransferase assays were performed by incubating DNA (7.6 nM for duplex II or 5.4 nM for duplex III) with [*Me*-³H]SAM (1.3 μM) (sp. radioactivity 85 Ci/mmol) and the appropriate amounts of enzyme in 1 × methylase buffer for 15 min. Subsequent manipulations of the reaction mixtures were as described above for the time-dependent reactions.

RESULTS

E. coli C5 MTase *M.EcoRII* [18,19] catalyses the methylation of the internal cytosine residues in the unmethylated or hemimethylated (i.e. one of the strands is methylated) sequence



To clarify the role of 5-azacytosine in the inhibition of C5 MTases, we synthesized a substrate containing a single site for *M.EcoRII*. The substrate was designed such that it would contain a single 5-azacytosine in the duplex, and this base would be the target for methyl transfer by the enzyme. Such a substrate was synthesized (duplex II, Table 1) by the polymerization of template–primer hybrid (duplex I, Table 1) in the presence of 5-azadCTP. In parallel reactions, a similar duplex containing normal cytosine at the target site was also synthesized (duplex III, Table 1).

When the polymerized products were end-labelled with ³²P and electrophoresed on a polyacrylamide gel, a new band of slower mobility than duplex I was observed (Figure 1). The polymerization product was susceptible to cleavage by *Bst*NI endonuclease (Figure 1). *Bst*NI cleaves DNA within the same sequence as *EcoRII*, but unlike *EcoRII* it is not inhibited by C5 methylation [20]. These results show that polymerization of the primer in duplex I created a site containing a CCWGG (W = A or T) sequence. Densitometric scanning of the autoradiograph presented in Figure 1 showed that only about 8% of duplex I was converted into duplex II. However, the efficiency of polymerization was improved to about 50% in subsequent experiments by increasing the concentration of 5-azadCTP in the reaction (results not shown). The products of the latter reaction were used for experiments described below. It should be noted that *Bst*NI digestion of the polymerized duplex created two fragments. However, the smaller fragment (8 bp) was electrophoresed off the gel and is not seen in Figure 1. Further, *Bst*NI endonuclease digests single-stranded DNA [17]. This accounts for the disappearance of much of the single-stranded DNA in Figure 1 after digestion with *Bst*NI.

Unlike the polymerization reactions with 5-azadCTP, reactions with dCTP were more than 90% complete (Figure 1). As expected, the mobilities of duplexes synthesized by using dCTP and 5-azadCTP were similar (Figure 1, and results not shown) and both the products were susceptible to *Bst*NI. We conclude that these polymerization reactions resulted in the synthesis of expected duplexes (III and II respectively).

To show that *M.EcoRII* forms a stable complex with duplex II, ³²P-end-labelled duplex II was incubated with excess purified *M.EcoRII* in the absence or presence of cofactor. The enzyme was denatured by adding SDS, and one half of each mixture was heated as described in the Materials and methods section. The resulting samples were electrophoresed in a polyacrylamide gel in

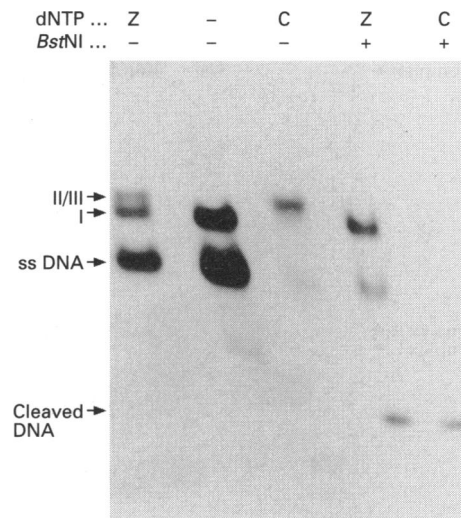


Figure 1 Synthesis of duplexes II and III

The polymerization reaction mixtures were electrophoresed on a polyacrylamide gel as described in the Materials and methods section. The dCTP derivative used to create the duplexes and the samples in which the duplexes were digested with *Bst*NI are indicated above the lanes: Z denotes 5-azadCTP, and C denotes normal dCTP. Equal amounts of labelled DNA (0.6 pmol) was loaded in each lane. The intensities of duplexes II and III are different, probably because the latter duplex was labelled 10 days earlier and it may not have been labelled as efficiently. The presence of significant amounts of single-stranded DNA in the first two lanes (marked as ss DNA) is caused by two factors. The expected temperature of melting of duplex I (42 °C) is only slightly above the temperature at which DNA polymerization was carried out, 37 °C. As a result, significant amounts of the two strands should exist as free DNA at this temperature. Secondly, although equal amounts of the two strands were used in the hybridization reaction, the template strand in the duplexes was expected to label more efficiently, because of its 5' overhang, than the primer strand. This is probably why the free template strand is seen in the autoradiogram, but not the primer strand. The positions of the various DNA molecules in the gel are indicated on the left of the Figure.

the presence of SDS. The results are shown in Figure 2. Although SDS-resistant complexes were found regardless of whether a cofactor was present in the reaction, SAM, but not SAH, stimulated the formation of a complex (Figure 2, compare lane 5 with lanes 7 and 9). In contrast, when duplex III was used in the reaction, no SDS-resistant complexes were observed in the presence or absence of SAM (results not shown). It should be pointed out that the residual duplex I or the single-stranded DNAs that exist in the preparation of duplex II do not form specific stable complexes with the enzyme (Figure 2, compare lane 3 with lanes 5, 7 and 9), and hence these DNAs are unlikely to interfere with the binding of the enzyme to duplex II (or to duplex III). These results suggest that *M.EcoRII* forms a covalent complex with azaC DNA and the formation of the complex is stimulated by SAM, in agreement with previously published reports [3,15].

When the SDS-resistant complexes formed in the absence of cofactor were subjected to heat, they completely dissociated to give rise to free DNA (Figure 2, lane 6). The complexes formed in the presence of SAH were also largely dissociated by heat (Figure 2, lane 10). In contrast, the complexes formed in the presence of SAM were resistant to heating (Figure 2, lane 8). The simplest interpretation of these results is that methyl transfer to azaC DNA makes it heat-resistant. It is possible that heat-induced elimination of a proton at N-5 is energetically much easier than the elimination of a methyl group (Scheme 3a). Presumably, elimination of the proton is thermodynamically unfavourable at lower temperatures.

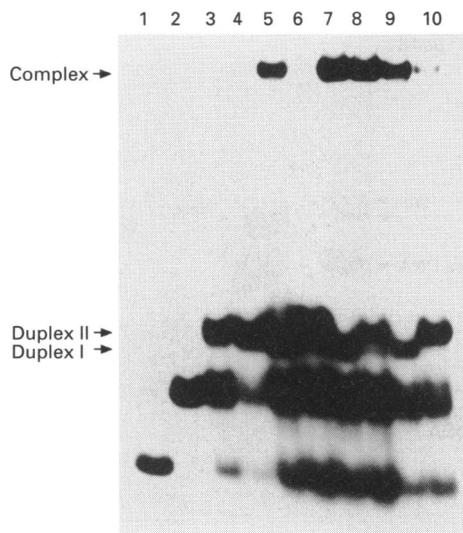
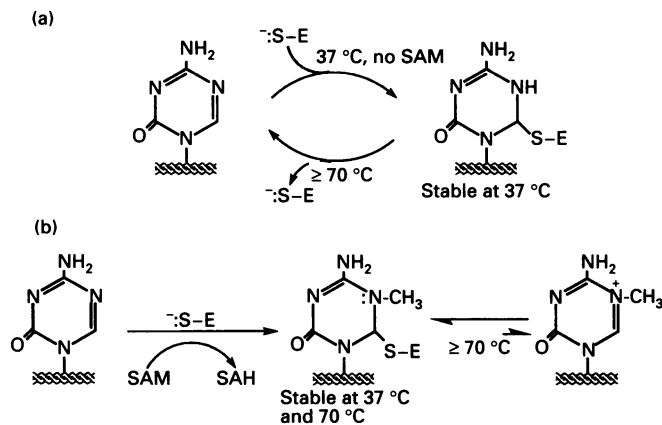


Figure 2 Covalent complex-formation between *M.EcoRII* and 5-azacytosine-containing DNA

Autoradiograph of a gel containing ^{32}P -labelled DNA is shown. The reaction conditions for the formation of the complexes are described in the Materials and methods section, except for the primer:template ratio, which was 2:1 in these experiments. Lanes: 1, 15-mer primer used for the synthesis of duplex I; 2, 25-mer template used for the synthesis of duplex I; 3 and 4; duplex II; 5 and 6, duplex II with MTase; 7 and 8, duplex II with MTase and SAM; 9 and 10, duplex II with MTase and SAH. Samples in lanes 4, 6, 8 and 10 were heated at 70°C before loading on the gel. The positions of the complexes and of the free DNAs are indicated by arrows.



Scheme 3 Revised mechanism of the inhibition of C5 MTases by 5-azacytosine in the absence (a) and presence (b) of SAM

— : S-E denotes C5 MTase.

The methyl transfer to duplex II in these reactions was quantified by mixing unlabelled duplex II with *M.EcoRII* and [$Me\text{-}^3\text{H}$]SAM. The reaction products were separated from free labelled SAM by spin-dialysis, and the amount of label incorporated into DNA was quantified by scintillation counting. The methyl-group incorporation measured by this assay was compared with the amount of duplex II calculated by gel densitometry. It was found that 0.11 pmol of methyl groups was transferred to 0.18 pmol of DNA. As one methyl group is expected to transfer to each DNA molecule, this represents 61 %

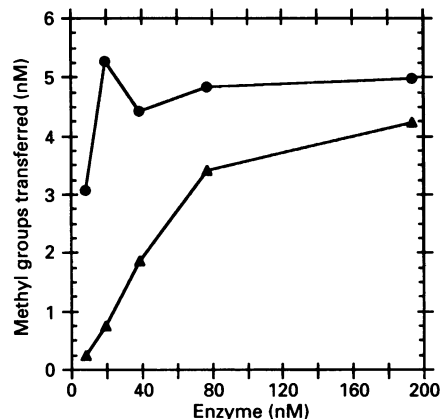


Figure 3 Dependence of methyl transfer to 5-azacytosine on enzyme concentration

The details of the assay are described in the Materials and methods section: \blacktriangle , duplex II; \bullet , duplex III. All the values reported here are averages of data from two separate experiments, except for the values at the lowest and the highest concentration of duplex II. In these two cases, results from one experiment are reported.

of the maximum expected methyl transfer. The discrepancy between the expected and the observed results either could be due to an overestimation of the duplex product, or could be the result of some rearrangements in the incorporated 5-azacytosine due to its reactivity. For these reasons, the concentrations of duplex II (and also of duplex III) used in subsequent experiments were calculated on the basis of the maximum amount of methyl groups that could be incorporated into it.

To demonstrate that incubation of duplex II with *M.EcoRII* and SAM results in the transfer of methyl groups to 5-azacytosine-enzyme complex, we separated the products of the reaction with [$Me\text{-}^3\text{H}$]SAM on a polyacrylamide gel. Gel pieces containing enzyme-DNA complexes or free DNA were excised, and the presence of ^3H was quantified by scintillation counting. About 80% of the radioactivity recovered from the gel was found in the complexed DNA (results not shown), consistent with the transfer of methyl groups to DNA. However, the remaining radioactivity was found in free DNA, suggesting that some of the methylated duplexes were released from the complex. It is possible that heat induces a type of β -elimination reaction, in which the lone pair of electrons on N-5 restores the double bond between N-5 and C-6 (Scheme 3b). However, the possibility of other rearrangements of the pyrimidine ring cannot be ruled out. In other experiments, the protein in the methylated complex was digested extensively with proteinase K, resulting in a complex of mobility intermediate between free DNA and the original complex. This complex was also found to contain [^3H]methyl groups (results not shown). This further supports the conclusion that the N-5 position of 5-azacytosine becomes methylated to form an irreversible covalent complex with the enzyme.

If methyl transfer to 5-azacytosine takes place, but the β -elimination step is blocked, then methyl transfer to azaC DNA should be limited by the amount of enzyme in the reaction when the substrate is in excess. To test this, we measured the amount of methyl transfer to duplex II at different enzyme concentrations. To ensure that all active enzyme molecules have the opportunity to react with the substrate, the reaction was done for 15 min. The enzyme is known to turn over in 30 s with normal DNA [3]. The results are shown in Figure 3. The increase of methyl transfer is

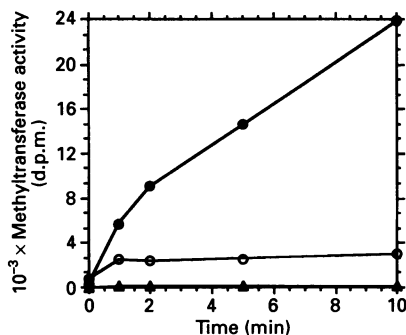


Figure 4 Time-dependence of methyl transfer to 5-azacytosine

The details of the assay are described in the Materials and methods section: ○, duplex II; ●, duplex III; ▲, duplex I.

linear with increasing enzyme concentration up to about 80 nM. Beyond this concentration the enzyme starts to be saturated. In an earlier study, Friedman [3] also found a linear relationship between the amount of the enzyme and the amount of complex between the enzyme and azaC DNA. If the enzyme were to transfer one methyl group to be the substrate and be inactivated, the ratio of enzyme:product should be 1:1 in the range of enzyme concentrations where the substrate is in excess. However, we found that this ratio was ~ 20:1 (see the linear range of the plot in Figure 3). We interpret these data to mean that only about 5% of the total enzyme present in the reaction was active. As expected, the linear relationship between the enzyme and product was not observed when duplex III was used as the substrate (Figure 3). Presumably the enzyme methylates normal DNA, turns over and methylates other substrate molecules.

The mechanism of inhibition of C5 MTases by 5-azacytosine predicts that, when the amount of enzyme is limiting, all enzyme molecules should become covalently linked to azaC DNA, and hence after the first round of the methylation reaction (about 30 s) further methyl transfer should not take place. To show that enzyme inactivation occurs after the first turnover of the reaction, we compared the time course of methyl transfer between normal DNA and azaC DNA. Methyl-transfer reactions by *M.EcoRII* to either duplex II or duplex III were performed by using an excess of DNA over active enzyme. Since only 5% of the enzyme may be active, the amount of active enzyme in each reaction was 0.0085 pmol, making the ratios of duplex II and duplex III to active enzyme to be 20:1 and 30:1 respectively. The background activity in this assay was determined with duplex I as the substrate. Little or no methyl transfer to this DNA was detected (Figure 4). When duplex II was the substrate, the enzyme was active only during the first 1 min of the reaction (Figure 4), suggesting that the enzyme remained linked to duplex II after it had transferred a methyl group. After 1 min there was little or no further methyl-group transfer to DNA. The maximum incorporation of methyl groups into duplex II was 0.012 pmol, in rough agreement with the estimated amount of active enzyme in the reaction. In contrast, when duplex III was the substrate, the enzyme remained active for at least 10 min (Figure 4). In a 10 min reaction, the enzyme is expected to turn over 20 times when methylating duplex III and to result in the incorporation of 0.17 pmol of methyl groups. This value is close to the amount of methyl groups actually incorporated into duplex III in 10 min (0.15 pmol). These results demonstrate that, whereas the C5 MTase methylates normal cytosine in DNA and turns over

several times, it transfers one methyl group to 5-azacytosine in DNA and becomes irreversibly inactivated.

DISCUSSION

Previous studies of the formation of covalent complexes between azaC DNA and C5 MTases have produced contradictory and ambiguous results regarding the role of SAM in the process. In one study [4], SAM was found not to affect binding of *HpaII* MTase to azaC DNA. In another study [15], binding of four C5 MTases, including *HpaII* MTase, to azaC DNA was found to be stimulated by SAM. In neither study was the transfer of methyl groups to 5-azacytosine in the site of methylation studied. This was because only a fraction of the DNA contained 5-azacytosine at the target site, and 5-azacytosine was present at sites in DNA that were not targets for methyl transfer.

We have used a substrate that contains a single 5-azacytosine at the site of methylation of *M.EcoRII* and used it to confirm that SAM stimulates binding of the enzyme to azaC DNA. Further, we have shown that the nature of the complex formed between C5 MTase and azaC DNA is different in the presence of SAM from that in the absence of SAM. However, unlike DNA substrates containing 5-fluorocytosine [12], the complexes formed by *M.EcoRII* with azaC DNA in the presence of SAM are not resistant to extreme denaturation conditions. The complexes also dissociate during long incubations and during manipulations such as gel elution. As a result, one cannot formally eliminate the possibility that *M.EcoRII* can form stable ternary complexes that include azaC DNA and SAM, but that either do not involve a covalent link between the enzyme and DNA or that do not involve methyl transfer to DNA. We think these to be unlikely, for several reasons. The complexes formed by *M.EcoRII* with azaC DNA in the presence of SAM are resistant to strong denaturing conditions and to proteolytic digestion. Such stable complexes are not formed in the absence of cofactor or in the presence of SAH (Figure 2). Further, when some of the complexes do dissociate during long storage or gel elution, the resulting free DNA contains methyl groups from SAM (see above).

Our results differ from those of Friedman [15] in one significant respect: whereas we found that complex-formation between azaC DNA and *M.EcoRII* occurred within the first 1 min of the reaction, Friedman [15] found that the complex-formation occurred over several minutes. It is possible that in the latter case the enzyme may transiently interact with 5-azacytosine present at sites that were not substrates for methylation and that this may have slowed down the kinetics of stable complex formation. In any case, our results strongly suggest that the inactivation of the enzyme occurs the first time the enzyme attacks its substrate.

Our results also suggest that, when a C5 MTase is inactivated by azaC DNA, two structures of the 'dead-end' product are possible: enzyme linked to methylated 5-azacytosine, or enzyme linked to protonated 5-azacytosine (Scheme 3). Covalent adducts formed by C5 MTases with azaC DNA *in vivo* are more likely to be of the former kind, because of the abundance of SAM in cells [21–23]. Hence, the kind of structure (protonated N-5) visualized by Santi et al. [1] is unlikely to occur *in vivo*.

The existence of two forms of complexes between 5azaC DNA and C5 MTases may be useful for identifying amino acid residues that are responsible for cofactor binding or that play a role in methyl transfer. For example, a mutant MTase defective in SAM binding, but able to initiate a nucleophilic attack on the cytosine, should form an SDS-resistant but heat-sensitive complex with azaC DNA in the presence of SAM. Thus 5-azacytosine may prove to be a useful analogue with which to study the structure-function relationships of C5 MTases.

We thank Dr. S. Friedman for providing 5-azadCTP and for critically reading the manuscript. The work presented here was supported by National Institutes of Health Grant GM45860 and by a Research Career Development Award HG00004 from the National Institutes of Health to A.S.B.

REFERENCES

- 1 Santi, D. V., Garrett, C. E. and Barr, P. J. (1983) *Cell* **33**, 9–10
- 2 Friedman, S. (1981) *Mol. Pharmacol.* **19**, 314–320
- 3 Friedman, S. (1985) *J. Biol. Chem.* **260**, 5698–5705
- 4 Santi, D. V., Norment, A. and Garrett, C. E. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6993–6997
- 5 McIntosh, L. P., Zielinski, W. S., Kalisch, B. W., Pfeifer, G. P., Sprinzl, M., Drahovsky, D., van de Sande, J. H. and Jovin, T. M. (1985) *Biochemistry* **24**, 4806–4814
- 6 Taylor, S. M. and Jones, P. A. (1982) *J. Mol. Biol.* **162**, 679–692
- 7 Taylor, S. M., Constantinides, P. A. and Jones, P. A. (1984) *Curr. Top. Microbiol. Immunol.* **108**, 115–127
- 8 Lu, L.-J. W. and Randerath, K. (1984) *Mol. Pharmacol.* **26**, 594–603
- 9 Osterman, D. G., DePillis, G. D., Wu, J. C., Matsuda, A. and Santi, D. V. (1988) *Biochemistry* **27**, 5204–5210
- 10 Chen, L., MacMillan, A. M., Chang, W., Ezaz-Nikpay, K., Lane, W. S. and Verdine, G. L. (1991) *Biochemistry* **30**, 11018–11025
- 11 Friedman, S. and Ansari, N. (1992) *Nucleic Acids Res.* **20**, 3241–3248
- 12 Wyszynski, M. W., Gabbara, S., Kubareva, E. A., Romanova, E. A., Oretskaya, T. S., Gromova, E. S., Shabarova, Z. A. and Bhagwat, A. S. (1993) *Nucleic Acids Res.* **21**, 295–301
- 13 Hanck, T., Schmidt, S. and Fritz, H.-J. (1993) *Nucleic Acids Res.* **21**, 303–309
- 14 Smith, S. S., Kaplan, B. E., Sowers, L. C. and Newman, E. M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4744–4748
- 15 Friedman, S. (1986) *Nucleic Acids Res.* **14**, 4543–4556
- 16 Huang, Y. and Friedman, S. (1991) *J. Biol. Chem.* **266**, 17424–17429
- 17 Gabbara, S. and Bhagwat, A. S. (1992) *J. Biol. Chem.* **267**, 18623–18630
- 18 May, M. S. and Hattman, S. (1975) *J. Bacteriol.* **122**, 129–138
- 19 Schlagman, S., Hattman, S., May, M. S. and Berger, L. (1976) *J. Bacteriol.* **126**, 990–996
- 20 Butkus, V., Klimasauskas, S., Kersulyte, D., Vaitkevicius, D., Lebionka, A. and Janulaitis, A. A. (1985) *Nucleic Acids Res.* **13**, 5727–5746
- 21 Adams, R. L. P. and Burdon, R. H. (1985) *Molecular Biology of DNA Methylation*, Springer-Verlag, New York
- 22 Greene, R. C., Hunter, J. S. V. and Coch, E. H. (1973) *J. Bacteriol.* **115**, 57–67
- 23 Satischandran, C., Taylor, J. C. and Markham, G. D. (1990) *J. Bacteriol.* **172**, 4489–4496

Received 26 July 1994/5 December 1994; accepted 12 December 1994