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Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine

(5-methylcytosine/5-azacytidine/*Hpa* II methylase)

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ABSTRACT **DNA containing 5-azacytosine (azaC) has previously been shown to be a potent inhibitor of DNA-cytosine methyltransferases. In this report, we describe experiments which demonstrate that azaC-DNA forms a covalent complex with *Hpa* II methylase, a bacterial enzyme that methylates the internal C of C-C-G-G sequences. The complex does not undergo detectable dissociation over at least 3 days and is stable to denaturation with $NaDodSO_4$. After extensive digestion of the complex with DNase and phosphodiesterase, gel filtration gave the methylase bound to approximately one equivalent of azaC; the digested complex had an apparent molecular weight similar to that of the native enzyme. Although prior treatment of azaC-DNA with *Hpa* II endonuclease had only a slight effect on binding of the methylase, treatment with *Msp* I endonuclease, which also cleaves at C-C-G-G sequences, resulted in a significant reduction in binding; this indicates that azaC residues in the recognition sequence of *Hpa* II are an important component in the covalent interaction of the methylase. However, since there was residual binding it is possible that azaC residues elsewhere in DNA also covalently bind to the methylase. These results provide an explanation of why azaC-DNA is such a potent inhibitor of cytosine methyltransferases and how the incorporation of such low levels of azaC into DNA can result in dramatic decreases in the methylation of cytosine. Finally, consideration of the probable catalytic mechanism of cytosine methylases and the chemical properties of azaC suggests that the inhibition is, at least in part, an active-site directed process and permits a proposal for the structure of the covalent complex.**

5-Methylcytosine, a minor base in the DNA of a variety of organisms, is formed by postreplicative methylation of DNA by *S*-adenosylmethionine (AdoMet) in reactions catalyzed by DNA-cytosine methyltransferases (DCMTases). In recent years, much evidence has been obtained which indicates that 5-methylcytosine residues in DNA play an important role in eukaryotic gene expression (for reviews see refs. 1 and 2). Consequently, there has been wide interest in the pyrimidine analog 5-azacytidine (azaCyd), which inhibits formation of 5-methylcytosine in DNA and results in dramatic effects on gene expression and cell differentiation (e.g., see refs. 3–9).

Current evidence indicates that the mechanism by which azaCyd causes decreased DNA methylation involves incorporation of 5-azacytosine (azaC) into DNA and subsequent inhibition of DCMTase. Incorporation of small amounts of azaC into DNA of mammalian cells results in a loss of DCMTase activity in extracts obtained from such cells (5, 10). Further, incubation of DNA containing azaC (azaC-DNA) with mammalian or bacterial DCMTases results in a very potent inhibition of enzyme activity (11, 12), but kinetic studies have not revealed the mechanism of inhibition. On the basis of the probable catalytic mechanism of DCMTases and known chemical properties of azaC, we recently speculated that the mechanism of inhibition involves covalent bond formation between a catalytic nucleophile of the enzyme and the reactive 6 position of azaC residues that replaced cytosine methylation sites in DNA (13). In this report we describe binding studies of azaC-DNA and *Hpa* II methylase, a bacterial DCMTase that methylates the internal C in C-C-G-G sequences. The experiments indicate that covalent complexes are formed that involve azaC residues within the recognition sequence of the methylase; however, covalent complexes may also involve azaC residues elsewhere in DNA. Our results, together with reported kinetic experiments (11, 12), explain how incorporation of small amounts of azaC into DNA can result in such dramatic decreases in the methylation of cytosine residues of DNA.

MATERIALS AND METHODS

Materials. *Hpa* II methylase and *Msp* I endonuclease were obtained from New England Biolabs. *Eco*RI methylase was a gift from H. Boyer. *Hae* III and *Hpa* II endonucleases were products of Bethesda Research Laboratories. Bovine pancreas DNase I was from Sigma, snake venom phosphodiesterase was from Boehringer Mannheim, and bacterial alkaline phosphatase was from Worthington. [6-^{3}H]azaCyd (1.6 Ci/mmol; 1 Ci = 37 GBq) and [*methyl*-^{3}H]thymidine (125 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA) and [*methyl*-^{3}H]AdoMet (62 Ci/mmol) was from New England Nuclear. AdoMet and azaCyd were obtained from Calbiochem–Behring. All other reagents were of the purest grade commercially available. Nitrocellulose BA85 membranes (0.45-μm pore diameter; 2.4 cm) were obtained from Schleicher & Schuell.

Enzyme Assays. Unless otherwise specified, endonucleases were used as suggested by the supplier. *Hpa* II methylase was assayed by the method of Yoo and Agarwal (14); 1 unit is defined as the amount of enzyme that catalyzes incorporation of 1 pmol of methyl groups into phage λ DNA in 1 hr at 37°C. From the reported molecular weight and specific activity of the purified enzyme (14), we calculate that 100 units is equivalent to about 1 pmol of enzyme.

DNA Preparations. *Escherichia coli* K-12 strain 993 (F^+, restriction-negative, methionine-requiring), obtained from B. Alberts, was grown in M9 medium containing methionine at 40 μg/ml at 37°C until the OD_{600} was 0.5–0.6. To individual 10-ml cultures was added (*i*) azaCyd at 25 μg/ml, (*ii*) 0.3 mCi of [6-^{3}H]azaCyd, (*iii*) 150 μCi of [^{3}H]thymidine, or (*iv*) 150 μCi of [^{3}H]thymidine plus azaCyd at 25 μg/ml. The culture containing [6-^{3}H]azaCyd was incubated for 30 min and others for 1 hr prior to harvesting. The DNA preparations were isolated by a modification of a reported procedure (15) with care taken to avoid exposure to acidic or basic condi-

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Abbreviations: AdoMet, *S*-adenosylmethionine; DCMTase, DNA-cytosine methyltransferase; azaCyd, 5-azacytidine; azaC, 5-azacytosine; azaC-DNA, DNA containing 5-azacytosine.

tions, which would result in degradation of azaC residues (16). Cells were washed with 2 ml of cold 150 mM NaCl/100 mM Na_2EDTA/20 mM Tris·HCl, pH 7.4, and resuspended in 1.0 ml of the same buffer. The suspension was treated with 100 μg of lysozyme for 15 min at room temperature and brought to 1% in $NaDodSO_4$. After 5 min, the mixture was extracted three times with equal volumes of phenol/chloroform (1:1, vol/vol) and nucleic acids were precipitated with 2 vol of ethanol. The precipitate was dissolved in 0.5 ml of 10 mM Tris·HCl, pH 7.4/1.0 mM EDTA and treated with previously boiled pancreatic RNase at 100 μg/ml for 1 hr at 37°C. The solution was brought to 0.1 M in NaCl and 0.2% in $NaDodSO_4$ and extracted twice with phenol/chloroform and the DNA was precipitated with 2 vol of ethanol. The precipitate was redissolved in 10 mM Tris·HCl, pH 7.4/1.0 mM EDTA, and the DNA was precipitated with 2-propanol, redissolved in the same buffer, and stored at −80°C.

The [6-^{3}H]azaC-DNA contained 2.7×10^4 dpm/μg, which, calculated on the basis of the specific activity of the analog and the 50% G+C content of *E. coli* DNA, corresponds to 1% substitution of the cytosine residues. To estimate the azaC content of azaC-DNA prepared with the unlabeled analog, the G+C content was determined by HPLC after digestion to the component nucleosides. A solution (300 μl) containing 10 μg of [^{3}H]thymidine-containing azaC-DNA ([^{3}H]dT-azaC-DNA), 50 mM Tris·HCl at pH 8.2, 10 mM $MgCl_2$, and 70 units of DNase I was incubated at 37°C. After 2 hr, 3.6 units of snake venom diesterase and 2 units of bacterial alkaline phosphatase were added and incubation was continued for 3 hr. The acid-soluble fraction was prepared and neutralized as previously described (17). HPLC using an Altex Ultra Sphere ODS column (5-μm diameter spheres; 4.6 × 250 mm) proceeded for 100 min with a linear gradient of 0–10% (vol/vol) methanol containing 5 mM sodium hexanesulfonate/5 mM acetic acid, pH 3.3, at a flow rate of 1.2 ml/min. This system effectively separates all major ribo- and deoxyribonucleosides; no ribonucleosides were detected, verifying the absence of RNA contamination. The deoxycytidine-to-deoxyguanosine ratio was 0.74, indicating that 26% of the cytosine residues were replaced by azaC.

Nitrocellulose Binding Assay. Nitrocellulose filter-binding assays of protein-DNA complexes were performed by a modification of reported procedures (see ref. 18). To reduce background binding, radioactive DNAs were first digested with *Hae* III endonuclease. Typically, solutions containing the radiolabeled DNA at 1–2 μg/ml and *Hae* III endonuclease at 30–40 units/ml in 50 mM Tris·HCl, pH 7.5/5 mM EDTA/10 mM 2-mercaptoethanol/5 mM $MgCl_2$/65 μg of bovine serum albumin per ml were incubated at 37°C for 1 hr. Using 50–100 μl of the digest, *Hpa* II methylase was added to give 130 units/ml and, where specified, AdoMet to give a final concentration of 5 μM; controls omitted the methylase. After incubation at 37°C for 1 hr, an aliquot was used to determine total radioactivity and duplicate aliquots (15–25 μl) were applied to nitrocellulose filters that were presoaked with 20 mM potassium phosphate, pH 7.4. The filters were washed under gentle suction with five 1-ml portions of the same buffer and dissolved in Aquasol II (New England Nuclear), and their radioactivities were measured.

$NaDodSO_4$ was removed from denatured *Hpa* II methylase-azaC-DNA complexes prior to nitrocellulose binding by column centrifugation through Sephadex LH20, which tenaciously binds the detergent (unpublished results). As described by Kreuzer and Alberts (19), a small hole was made in the bottom of a 1.5-ml Eppendorf tube (column tube) with a 27 gauge needle and a larger hole was made about one-third of the way from the top of another (carrier tube) with a 19 gauge needle. About 40 μl of silane-treated glass beads (0.17–0.18 mm diameter) was placed at the bottom of the column tube and overlayed with about 1 ml of Sephadex LH20 in 25 mM Tris·HCl, pH 7.4/30 mM EDTA. The column tube was placed in the carrier tube and centrifuged at setting 80 in a Clay Adams centrifuge for 2 min, giving a packed volume of about 0.6 ml. After the carrier tube had been replaced, up to 60 μl of sample containing 1% $NaDodSO_4$ was applied to the column tube and centrifuged as above, and aliquots of the eluate were analyzed by the nitrocellulose binding assay. The recovery of DNA and protein was at least 85%, and control experiments indicated that the $NaDodSO_4$ content in the eluant was less than 0.005% as determined spectrophotometrically (20).

RESULTS

Binding of *Hpa* II Methylase to azaC-DNA. In early experiments, nitrocellulose binding assays were performed with unmodified radioactive DNAs from *E. coli*. Although *Hpa* II methylase clearly increased the binding of azaC-DNA to nitrocellulose, the background binding of DNAs was unacceptably high, amounting to 20–30% of the radioactivity applied. We subsequently found that treatment of radiolabeled DNAs with restriction endonucleases reduced the background binding to acceptable levels of about 2–4% of the labeled material applied to the membranes. All nitrocellulose binding assays described below used DNAs that were previously digested with *Hae* III endonuclease, which cleaves at G-G-C-C sequences and should produce fragments of DNA with an average size of about 256 base pairs. Further, unless otherwise specified, binding experiments were performed with [^{3}H]dT-azaC-DNA in which approximately 25% of the cytosine residues were replaced by azaC.

Incubation of *Hpa* II methylase (130 units/ml) and [^{3}H]dT-azaC-DNA (1.5 μg/ml) at 37°C resulted in a rapid time-dependent increase in filter-bound radioactivity; about 50% of the maximal radioactivity was bound at 2 min and there was no significant change after 10 min. With [^{3}H]dT-azaC-DNA at 1.5 μg/ml and a 1-hr incubation at 37°C, the binding was linear with respect to the methylase up to about 80 units of enzyme per ml, after which saturation occurred. As shown in Table 1, with excess *Hpa* II methylase (130 units/ml; *ca.* 1.3 pmol/ml), about 50% of the azaC-DNA present in the incubation mixture (1.5 μg/ml) was bound to the enzyme after correction for background binding; five separate determinations under similar conditions gave 49 ± 3% (mean ± SD) of bound azaC-DNA, with a range of 45–52%. The presence of AdoMet had no effect on the amount of azaC-DNA bound. As described below, complex formation requires the presence of azaC in DNA. Since many of the *Hae* III frag-

Table 1. Binding of [^{3}H]dT-azaC-DNA to *Hpa* II methylase

Methylase	[^{3}H]dT-azaC-DNA bound	
	dpm	%*
I. Before denaturation		
Hpa II	5850	56
Hpa II + AdoMet	5590	54
—	430	4
II. After denaturation		
Hpa II	4460	43
Hpa II + AdoMet	4870	47
—	300	3

Solutions (100 μl) contained *Hae* III endonuclease-digested [^{3}H]dT-azaC-DNA (0.15 μg; *ca.* 52,000 dpm), *Hpa* II methylase (13 units; *ca.* 0.13 pmol), and, where specified, 5 μM AdoMet. After incubation at 37°C for 1 hr, the total radioactivity in 20 μl was measured and 20 μl was assayed by nitrocellulose binding. The remainder was adjusted to 1% $NaDodSO_4$ and incubated at 37°C for 1 hr; after removal of the denaturant, 20-μl aliquots were assayed by nitrocellulose binding.

*Calculated on the basis of 10,400 dpm/20-μl aliquot.

ments should not contain azaC in the sequence recognized by the methylase, it is not unexpected that all of the DNA was not retained on the filter. Further, the values obtained may be artifactually low because we do not know how efficiently the filters retain the complex. When the *Hpa* II methylase-azaC-DNA complex was treated with 1% $NaDodSO_4$ for 1 hr at 37°C, most of the complex could be isolated on nitrocellulose filters after removal of the detergent; the small decrease in binding may be attributed to the manipulations required to remove the denaturant. As with the native complex, the presence of AdoMet during formation of the complex had an insignificant effect on the stability of the denatured complex. Prior treatment of *Hpa* II methylase with 1% $NaDodSO_4$ resulted in a complete loss of enzyme activity and prevented binding to azaC-DNA.

The interaction between *Hpa* II methylase and azaC-DNA appears to be a specific one. The requirement for azaC-DNA was established by demonstrating that only background radioactivity of about 3% was bound to nitrocellulose membranes when binding experiments were performed with [^{3}H]thymidine-containing DNA (1.5 μg/ml) not possessing azaC residues and *Hpa* II methylase (130 units/ml). Likewise, evidence for the requirement of the cytosine methylase was obtained by demonstrating that incubation of [^{3}H]dT-azaC-DNA (1.5 μg/ml) with the DNA-adenine methyltransferase, *Eco*RI methylase (up to 5 pmol/ml), for 1 hr at 37°C resulted in only background binding to nitrocellulose.

Stability of the Native *Hpa* II Methylase-azaC-DNA Complex. Experiments were performed to determine the rate of dissociation of *Hpa* II methylase-azaC-DNA complexes. A complex was formed as previously described, using 20 units of methylase and 0.2 μg of [^{3}H]dT-azaC-DNA in 150 μl; nitrocellulose membranes retained 3000 dpm/10-μl aliquot of this solution. A 30-fold excess of unlabeled azaC-DNA (6 μg in 15 μl) was added to 130 μl of this solution and 10-μl aliquots were removed at intervals and assayed for protein-bound radioactivity. Over a period of 3 days there was no change in bound radioactivity (2800 ± 200 dpm/10 μl; mean ± SD, n = 10), indicating that there was no detectable dissociation of the complex. A similar experiment was performed in which the complex was formed in the presence of 5 μM AdoMet before excess unlabeled azaC-DNA was added; as before, there was no change in bound radioactivity (3100 ± 260 dpm/10 μl; n = 10) over a period of 3 days. When the labeled and unlabeled azaC-DNA samples were premixed prior to addition of *Hpa* II methylase, the bound radioactivity was 150 dpm/10 μl, which is what would have been observed if all of the enzyme had dissociated from the initially bound complex and recombined with the total azaC-DNA of lower specific activity.

Nuclease Digestion of the Complex. A 50-μl solution of the standard binding buffer containing 65 units (*ca.* 0.65 pmol) of *Hpa* II methylase and 7.0 μg (1.9×10^5 dpm) of unrestricted [6-^{3}H]azaC-DNA was incubated for 3 hr at 37°C; in the control, *Hpa* II methylase was omitted. To each solution was added a solution (100 μl) containing 15 mM $MgCl_2$, 30 mM Tris·HCl at pH 8.3, 21 units of pancreatic DNase I, and snake venom phosphodiesterase at 20 μg/ml. After 18-hr incubation at 37°C, the solution was subjected to HPLC on a gel permeation column containing 3000 SW (TSK; Varian, CA). From the 0.50-ml fractions collected, 200 μl of each was used to measure total radioactivity and 200 μl was assayed for protein-bound radioactivity by adsorption to nitrocellulose. As shown in Fig. 1, the eluate from the digested *Hpa* II-[6-^{3}H]azaC-DNA complex shows a peak of radioactivity corresponding to an apparent M_r of about 45,000. The total radioactivity in this peak was 2200 dpm, which corresponds to 0.62 pmol of [6-^{3}H]azaC. Most (65%) of this radioactivity could be adsorbed on nitrocellulose, indicating it was bound to protein; the incomplete adsorption is attributable to the filtration efficiency, which is rarely 100% (18). The radioactivity that eluted after the macromolecular peak corresponds to low molecular weight nucleotides of [6-^{3}H]-azaC and did not bind to nitrocellulose. In the control that omitted *Hpa* II methylase, the only radioactivity eluting from the column corresponds to low molecular weight nucleotides of [6-^{3}H]azaC.

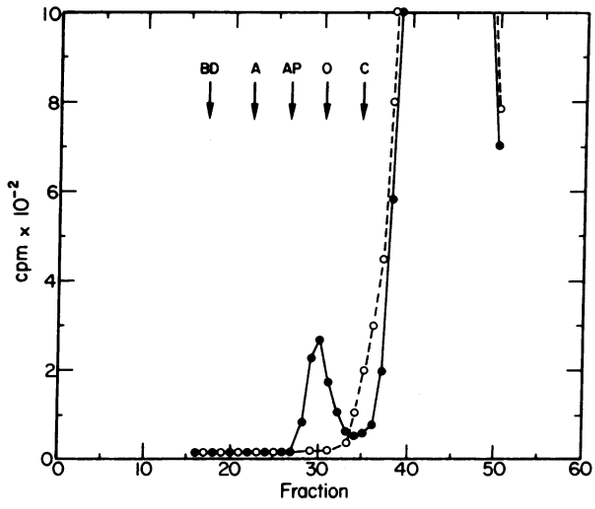


FIG. 1. Gel filtration of the *Hpa* II methylase-[6-^{3}H]azaC-DNA complex and [6-^{3}H]azaC-DNA after digestion with DNase I and phosphodiesterase. The digests were chromatographed on a 0.75 × 50 cm TSK 3000 SW column equilibrated and eluted, at 1.0 ml/min, with 50 μM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate (Tes), pH 7.4/1 mM EDTA/5 mM dithiothreitol/0.3 M KCl, collecting 0.50-ml fractions. ●, Radioactivity in the eluate of the digest of the *Hpa* II methylase-[6-^{3}H]azaC-DNA complex; ○, radioactivity in the eluate of the digest of [6-^{3}H]azaC-DNA. The arrows show the position of elution of calibration standards: BD, blue dextran ($M_r \approx 2 \times 10^6$); A, aldolase ($M_r$ = 158,000); AP, alkaline phosphatase (M_r = 86,000); O, ovalbumin (M_r = 43,000); C, chymotrypsinogen (M_r = 26,000).

Effect of *Hpa* II and *Msp* I Endonuclease Digestion of azaC-DNA. An attempt was made to determine whether *Hpa* II methylase was bound to azaC residues in the C-C-G-G methylation sequence or to other azaC residues of azaC-DNA. If the former were the case, hydrolysis with *Hpa* II and *Msp* I restriction endonucleases, which both cleave at C-C-G-G sequences, should reduce binding of the methylase to azaC-DNA. In the experiment shown in Table 2, 0.15 μg [^{3}H]dT-azaC-DNA was digested with 10 units of the specified endonuclease in 0.1 ml of the appropriate buffer for 1 hr at 37°C

Table 2. Binding of *Hpa* II methylase to endonuclease-digested [^{3}H]dT-azaC-DNA

Endonuclease	*Hpa* II methylase	[^{3}H]dT-azaC-DNA bound	
		dpm	%*
Hae III	+	4230	49
Hae III	−	320	4
Hpa II	+	3730	43
Hpa II	−	300	3
Msp I	+	2820	32
Msp I	−	260	3

Endonuclease digestions were performed as described in the text, after which 6.7 units of *Hpa* II methylase was added to 50 μl of the digest and an equal volume of water to the remaining 50 μl. After incubation at 37°C for 1 hr, 15 μl was used to measure total radioactivity and two 15-μl aliquots were assayed by nitrocellulose binding.

*Calculated on the basis of 8700 dpm/15-μl aliquot.

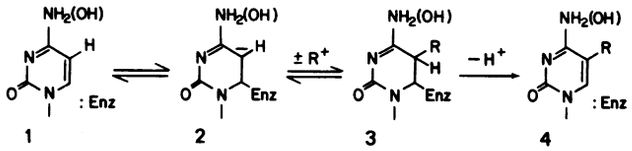
prior to assay for *Hpa* II methylase binding. Restriction of azaC-DNA with *Hpa* II endonuclease resulted in only a small decrease in binding compared to the control *Hae* III endonuclease digest. *Msp* I endonuclease had a greater effect, reducing binding to about 30%, but did not completely eliminate binding. In a similar experiment, the [^{3}H]dT-azaC-DNA was incubated overnight with a large excess of *Hpa* II endonuclease (100 units/ml) and *Msp* I endonuclease (60 units/ml) prior to binding with *Hpa* II methylase. The results were essentially identical to those in Table 2; 41% of the *Hpa* II digest and 34% of the *Msp* I digest bound to the methylase.

DISCUSSION

AzaC-DNA has been shown to be a potent inhibitor of DCMTase activity from mammalian and bacterial sources (11, 12), but kinetic studies have not revealed the mechanism of inhibition. In the present study, we have utilized direct binding assays to investigate the interaction of azaC-DNA with *Hpa* II methylase, a bacterial DCMTase that methylates the internal cytosine of C-C-G-G sequences. The major conclusion is that the enzyme forms a covalent bond with azaC residues of DNA. The complexes formed between radioactive azaC-DNA and *Hpa* II methylase can conveniently be isolated on nitrocellulose membranes, and the enzyme does not form isolable complexes with DNA not possessing azaC. As in the inhibition of DCMTase activity by azaC-DNA (11, 12), formation of these complexes does not require the presence of the cofactor AdoMet. The interaction appears to be specific for the cytosine methylase since we could not isolate complexes of azaC-DNA and *Eco*RI methylase, a DNA-adenine methyltransferase, and azaC-DNA does not inhibit the DNA-adenine methylase of *E. coli* K-12 (12). The evidence that azaC-DNA forms a covalent complex with *Hpa* II methylase is as follows. First, there is no detectable dissociation of the complex after as long as 3 days. Second, the complex formed in the presence or absence of AdoMet is stable towards denaturation with NaDodSO$_4$. Third, after extensive DNase/phosphodiesterase digestion of a complex in which 1% of the cytosine residues of DNA were replaced by [6-^{3}H]azaC, gel filtration gave a single peak of protein-bound radioactivity that possessed approximately 1 mol of azaC per mol of enzyme. Although we do not know the number of nucleotides remaining in the digested complex, it eluted with an apparent M_r of 45,000, which is within experimental error of the reported molecular weight of *Hpa* II methylase (14).

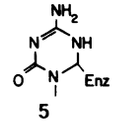
An important question is whether DCMTases are bound to azaC residues in specific recognition sequences or to azaC residues elsewhere in azaC-DNA. In the former case, cleavage of recognition sequences with specific restriction endonucleases should proportionately reduce binding of the methylase to azaC-DNA. It has been reported that restriction of azaC-DNA with *Eco*RII and *Hpa* II endonucleases partially reduces the inhibitory effect towards the corresponding DCMTases (12); the incomplete reduction of inhibition was attributed to resistance of azaC-DNA towards the endonucleases used. In similar experiments described here, treatment of azaC-DNA with *Hpa* II endonuclease had only a slight effect on the binding of *Hpa* II methylase. However, treatment with *Msp* I endonuclease, which also cleaves at C-C-G-G sequences, resulted in a 40% reduction in binding. Thus, we may conclude that azaC residues in *Hpa* II recognition sites are an important component in the covalent interaction with the methylase. The residual binding of the methylase to *Msp* I restriction digests may be due to incomplete cleavage of C-C-G-G sequences containing azaC. Indeed, it has been demonstrated that a variety of base analogs adjacent to the phosphodiester bonds cleaved by restriction endonucleases can dramatically reduce the activity of these enzymes (21). However, since even extensive treatment of azaC-DNA with *Msp* I endonuclease did not completely eliminate binding, we cannot rule out the possibility that azaC residues elsewhere in DNA may be bound to the methylase.

Consideration of the probable catalytic mechanism of DCMTases and the chemical properties of azaC permits the proposal of a reasonable mechanism for the covalent interaction of these enzymes with azaC residues contained in methylation sites of appropriate recognition sequences of DNA. Enzymes, such as DCMTases, that catalyze electrophilic substitution at the 5 position of pyrimidines are believed to proceed by the mechanism depicted in Scheme 1 (for reviews see refs. 22 and 23).



SCHEME 1.

Here, a nucleophilic catalyst of the enzyme adds to the 6 carbon of the heterocycle to activate what is otherwise an inert 5 carbon for reaction with an electrophile (R^+). Subsequent abstraction of the proton at the 5 position and β elimination provides the 5-substituted pyrimidine and active enzyme. Enzymes that catalyze such reactions can be potently inhibited by certain substrate analogs that, after formation of specific reversible complexes, react with the nucleophilic catalyst to form stable covalent adducts analogous to **3**. A necessary chemical feature of such inhibitors is that the 6 carbon be reactive towards nucleophiles found in proteins. Significantly, the 6 carbon of azaCyd readily reacts with nucleophiles to form 5,6-dihydropyrimidine adducts that, depending on conditions, may be quite stable or may undergo subsequent conversions leading to expulsion of the 6 carbon and formylation of the attacking nucleophile (16, 24). From the above, it is reasonable to propose that azaC-DNA inhibition of DCMTases proceeds, at least in part, by an active-site directed process. That is, once the enzyme binds to a recognition sequence containing azaC in a methylation site, the catalytic nucleophile would add to the 6 carbon as in the normal enzymic reaction to provide the covalent adduct **5**.



In principle, **5** could rearrange to give an inactive formylated enzyme, but this is not the case in the present work since the covalent complexes isolated contain azaC-DNA.

As previously discussed, it is possible that azaC residues other than those in methylation sites of DNA may also form covalent bonds with DCMTases. Considering the high reactivity of the 6 position of azaC, **5** would also represent the most reasonable structure for these putative complexes, but their formation could not be explained by the active-site directed process described above. Since restriction/modification enzymes are so specific, should such complexes exist, knowledge of their mechanism of formation and sequence specificity could reveal important information regarding methylase–nucleic acid interactions and recognition. Resolution of this issue will require more in-depth studies using azaC-DNAs of defined structure and analysis of DNA sequences flanking the covalently bound methylase.

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