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Binding of the *Eco*RII methylase to azacytosine-containing DNA

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ABSTRACT

Binding of DNA(cytosine-5)methyltransferases to azacytosine containing DNA is stimulated by the presence of S-adenosyl-methionine or its analogs sinefungin or S-adenosyl-L-homocysteine. Methylation of the DNA is therefore not necessary for binding to occur. There is no relationship between the affinity of the analog for the EcoRII enzyme and its ability to stimulate binding.

The DNA-enzyme complex partially dissociates on incubation in 0.1% sodium dodecyl sulfate and 0.5 M ammonium acetate. Some of this DNA could again form a tight complex with enzyme, indicating that DNA-enzyme complex formation is reversible.

Binding occurs when the second cytosine in the sequence CCAGG is substituted by azacytosine. This is the cytosine that would normally be methylated by the enzyme. The binding is therefore due to specific interaction of the methylase with azacytosine at the site it would normally methylate.

INTRODUCTION

5-azacytosine-containing DNA (azaC-DNA) inhibits bacterial DNA-(cytosine-5)methyltransferases (1). The *E. coli* EcoRII modification methylase, which is a cytosine methylase, is inhibited irreversibly. Irreversibility is defined in this instance by the inability to regenerate enzyme activity by dilution of the enzyme-DNA complex, or by digesting the DNA with either micrococcal nuclease or DNase I after the enzyme complex is formed. Both these enzymes destroy the inhibitory ability of the DNA if they are incubated with the DNA before addition of the methylase (1).

Azacytosine-containing DNA fragments end labelled with ^{32}P bind to the purified EcoRII methylase. For binding to occur the DNA must also contain the sequence recognized by the EcoRII methylase, the sequence CCA/TGG (2). It is the second C from the 5' end that is methylated by this enzyme (3). Binding can be demonstrated by polyacrylamide gel electrophoresis of the complex

after treatment with either 1% SDS or 6 M urea (2). Formation of the complex was stimulated by the presence of S-adenosyl-methionine (AdoMet), the methyl donor in the methylation reaction.

In this paper we explore the mechanism of this reaction. We provide evidence that the stimulation of binding caused by S-adenosylmethionine is different from its role in the catalytic reaction, where it serves as a methyl donor. This applies not only to the EcoRII methylase but to several other procaryotic methylases studied. Additional evidence is presented that the EcoRII enzyme binds specifically to the azacytosine in the DNA that would normally be methylated. Furthermore, we show that the enzyme-DNA complex can be dissociated, and that the DNA can again form a complex with the EcoRII enzyme. These data are discussed with regard to the mechanism of the binding reaction.

MATERIALS AND METHODS

[γ^{32}P]ATP was purchased from ICN Chemical and Radioisotope Division, Irvine, CA; MspI, HpaII, and HhaI, methylases were purchased from New England Biolabs; HinfI and AluI endonucleases were purchased from Bethesda Research Laboratories; EcoRII methylase was prepared from E. coliHB101 containing the methylase gene cloned from plasmid N3 into the EcoRI site of pBR322 (2). Calf intestine alkaline phosphatase and 5-azacytidine were purchased from Sigma Chemical Company. E. coli Sϕ441 (cdd-5 upp-11 relA1 metB1 rpsL254) was from Dr. Barbara Bachman.

Preparation of azaC-DNA fragments- DNA fragments were prepared from plasmid pBR322 grown in E. coli Sϕ441 as previously described (2). Briefly, cells were grown in minimal A medium (4) supplemented with 40 ug/ml DL-methionine at 37°C with continuous agitation. When the culture reached an absorbance of 0.5 at 550 nm, it was treated with 20 ug/ml 5-azacytidine. After a 3 h incubation an additional 20 ug/ml of drug was added and the incubation was terminated after 3 h. The plasmid was extracted from cleared lysates of cells (5) and purified by Sephadex G-100 chromatography, equilibrium density centrifugation in CsCl, and finally by centrifugation through a 5-20% sucrose gradient containing 1 M NaCl and 0.1 M potassium phosphate, pH 7.2, in an SW27 rotor at 27,000 rpm, 18°C, for 15 h. DNA, 20 ug, was digested overnight, with 20 units of HinfI endonuclease and dephospho-

rylated with calf intestine alkaline phosphatase (6). The DNA was electrophoresed through an 8% polyacrylamide gel prepared in TBE buffer (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA) at 5 V/cm for 15 h. The gel was stained with ethidium bromide and the appropriate bands were eluted by diffusion (7). DNA fragments were end-labeled with ^{32}P as described by Maxam and Gilbert (7). Fragments were further digested with the appropriate endonuclease and purified by gel electrophoresis. The pBR322 nucleotide positions of the DNA fragments used in this work are: 32 bp AvaI-HhaI, 1424-1456; 221 bp HinfI, 1303-1524; 154 bp HinfI 851-1005; and the 67 bp MspI fragment, 3482-3549.

The azacytosine content of the DNA fragments was determined by hydrolyzing the end labeled fragments in 1 M piperidine and running the digests on a sequencing gel. The per cent of the total radioactivity in the band of lowest molecular weight was determined for three fragments and averaged.

DNA-Enzyme Binding- The reaction mixture contained sodium chloride, 100 mM; dithiothreitol, 20 mM; Tris-HCl, pH 8, 50 mM; bovine serum albumin, 500 ug/ml; EDTA, 1 mM; DNA as indicated, 500-2000 cpm; and enzyme in a total of 30 ul. The reactions were incubated at 28°C for the times indicated. SDS, 12.5%, 1 ul, was added followed by 4 ul of 50% glycerol containing 0.1% bromphenol blue. The reaction mixture was then loaded on an 8% polyacrylamide gel prepared in TBE buffer and run as described in the text. The gels were then autoradiographed with one Dupont lightening plus intensifying screen at -70°C. Individual bands were cut out of the gels and counted in a liquid scintillation counter.

The enzyme concentration was determined by titration with ^{32}P labeled DNA. The DNA-enzyme complex was isolated by gel electrophoresis and the Cerenkov radiation determined in a Beckman Scintillation counter.

Preparation of the azaC-DNA-EcoRII methylase complex-A 32 bp AvaI-HhaI end labeled DNA fragment was prepared. The DNA, 32,000 cpm, was incubated as described above with AdoMet, 54 uM, and 4.4 units of enzyme in a volume of 0.8 ml for 1 h at 28°C, except that the albumin concentration used was 100 ug/ml. E. coli B DNA, 5 ug, was added and the mixture precipitated with 0.1 volume 3 M

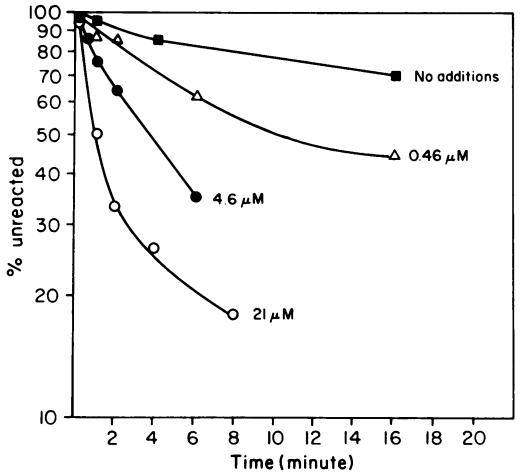


Figure 1. The effect of AdoMet on the binding of the EcoRII methylase to azaC-DNA. Reactions were performed with 2.3 fmoles of a 201 bp HinfI fragment as described in "Methods" in the presence of the indicated concentrations of AdoMet. Total reacted DNA was determined with excess enzyme after 60 min of incubation. EcoRII enzyme, 0.007 units, was used in the reactions. The enzyme was present in 6 fold excess.

sodium acetate, pH 7, and 2.5 volumes ethanol and frozen at -70° C for 1 h. The precipitate was collected by centrifugation at 10,000 x g for 10 min and dissolved in 100 ul buffer containing Tris-HCl, 10 mM, pH 8; EDTA, 1 mM; and SDS, 0.4%; and run on an 8% polyacrylamide gel for 2 h. at 10 volts/cm. The radioactive bands were detected by autoradiography, cut out, and eluted overnight by diffusion at 37°C as described by Maxam and Gilbert (7) and concentrated by precipitation with ethanol.

Enzyme assay-The standard assay measured the transfer of [^{3}H] methyl groups to E. coli B DNA from AdoMet[methyl-^{3}H] as previously described (1). One unit of enzyme activity is defined as the amount of enzyme that transfers one pmole of methyl groups per minute from AdoMet to DNA. The assay for K_m and K_i determination was as described (2).

RESULTS

Effect of AdoMet and its analogues on the formation of the enzyme-DNA complex- AdoMet stimulated reaction of the EcoRII enzyme with azaC-DNA fragments. As shown in figure 1 both the rate and the extent of complex formation was stimulated by the

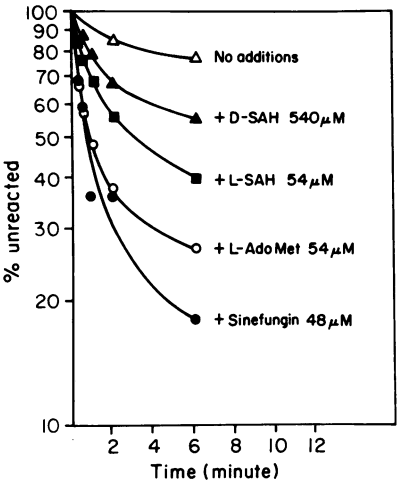


Figure 2. The effect of AdoMet analogues on the binding of the EcoRII methylase to azaC-DNA. A time course of the reaction of the 201 bp HinfI fragment with the EcoRII methylase in the presence of AdoMet analogues was carried out as described in figure 1 and "Methods". L- or D-SAH, L- or D-S-adenosyl-homocysteine.

addition of AdoMet. The reaction did not follow pseudo-first order kinetics beyond the first two minutes in the presence of excess enzyme. This was not due to the destruction of either enzyme or DNA during the incubation. A dissociation constant for AdoMet of 9.9 uM was calculated from the initial rates of complex formation presented in figure 1 from a plot of 1/V vs 1/s. This is close to the K_m of the enzyme for AdoMet in the methylation reaction, which was 6.2 uM at 28°C.

To determine if stimulation of the rate of complex formation were due to methylation of the DNA by the enzyme we tested several AdoMet analogues for their ability to stimulate the binding reaction. All the analogues tested stimulated the reaction. Figure 2 presents the time course of the reaction with 3 analogues as well as with AdoMet, at concentrations that are 8-70 times their respective K_i's. The K_i's are given in table I. There is no relationship between the K_i of the analogues for the enzyme in the methylation reaction and their ability to stimulate the rate of complex formation. 5'-S-isobutyl-5'-deoxyadenosine (SIBA) could

Table 1.
The Affinity of AdoMet Analogues
for the EcoRII Methylase[a]

Analogue	Ki μM
Sinefungin	4.3
L-S-adenosylhomocysteine (L-SAH)	0.83
D-S-adenosylhomocysteine (D-SAH)	45.8
5'S-isobutyl-5'-deoxyadenosine (SIBA)	180

[a]The inhibitory constants were determined from Lineweaver Burk plots of the methylation of E.coli B DNA with AdoMet, S-[methyl-^{3}H]. The K_m for AdoMet was 6.2 μM.

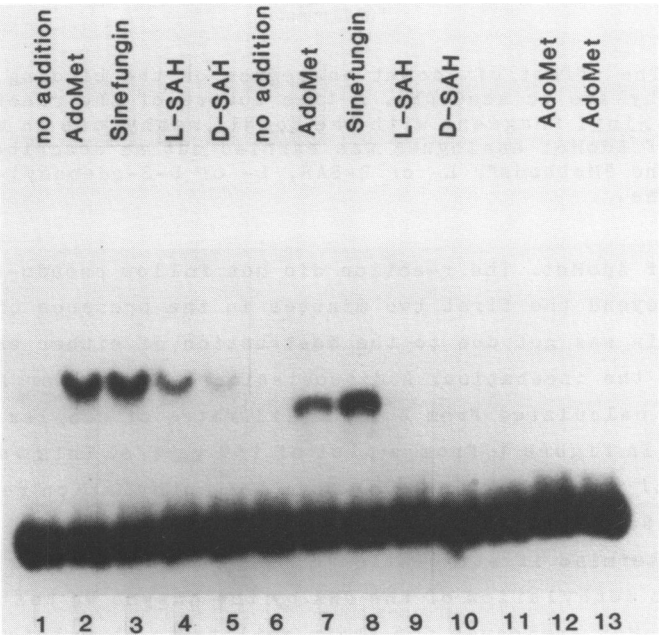


Figure 3. The effect of AdoMet and its analogues on the binding of the HpaII and MspI methylases to an azaC-DNA fragment. A 165 bp HinfI fragment was incubated with either 0.006 units of MspI or 0.014 units of HpaII methylase with either AdoMet or the indicated analogue, 57 uM, for 5 min as described in "Methods". Lanes 1-5, and 12, MspI; lanes 6-10, and 13, HpaII; lane 11, without enzyme; lanes 1-11, azaC-DNA; lanes 12 and 13, control fragment that does not contain 5-azacytosine.

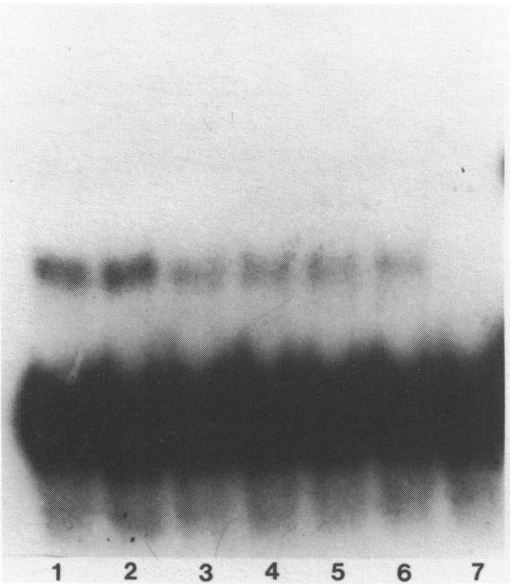


Figure 4. The effect of AdoMet and its analogues on the binding of the HhaI methylase to an azaC-DNA fragment. A 67 bp MspI fragment was incubated with 0.20 units of HhaI methylase with either AdoMet or the indicated analogue, 64 uM, for 5 min as decribed in "Methods" prior to electrophoresis for 2.5 h. Lane 1, AdoMet; lane 2, sinefungin; lane 3, L-S-adenosylhomocysteine; lane 4, D-S-adenosylhomocysteine; lane 5, 5'-S-isobutyl-5'-deoxyadenosine; lane 6, no addition; lane 7, without enzyme.

not be tested at 10 times its K_i. Binding of DNA fragments not containing azacytosine to the EcoRII methylase could not be detected by this assay even in the presence of 64 uM sinefungin and in the absence of SDS (data not shown).

Effect of AdoMet and its analogues on binding of MspI, HpaII and HhaI methylases to azaC-DNA fragments- In order to determine if the stimulation of binding by adenosylmethionine was peculiar to the EcoRII methylase or was a general property of these enzymes we tested several other procaryotic DNA(cytosine-5)-methylases.

MspI and HpaII are isoschizomers that methylate the sequence CCGG. A 154 bp HinfI fragment containing one such site was incubated with these methylases. The reaction of these methylases differed from the EcoRII methylase in that no reaction could be

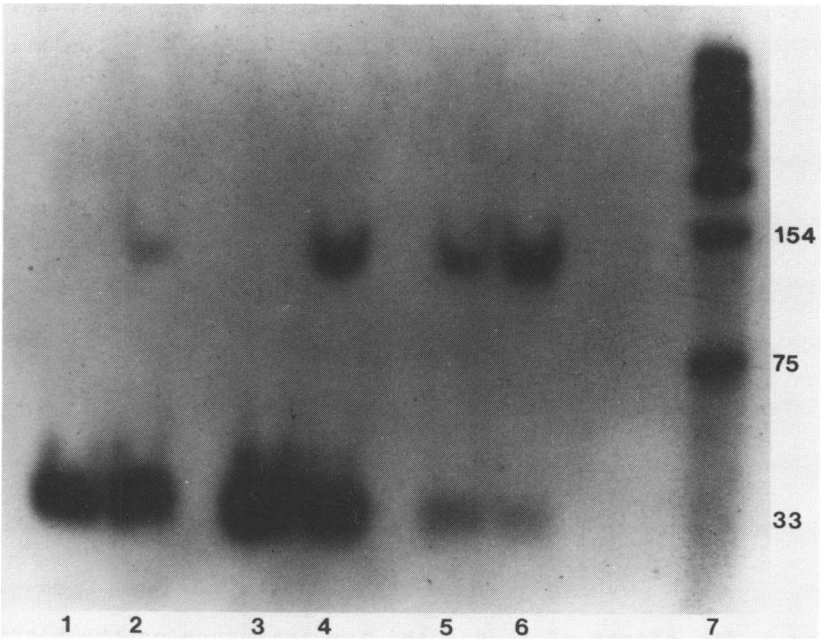


Figure 5. Electrophoretic analysis of the purified azaC-DNA-EcoRII methylase complex. Enzyme was incubated with a 32 bp azaC-DNA fragment and the enzyme-DNA complex separated from unreacted DNA as described in "Methods". The radioactive bands were cut out and eluted from the gel. Aliquots were then incubated with AdoMet, 57 uM, with or without enzyme, 0.04 units, in the standard incubation mixture for 1 h prior to analysis by polyacrylamide gel electrophoresis. Lanes 1 and 2, uncomplexed DNA, 600 cpm; lanes 3 and 4, DNA not previously treated with enzyme, 900 cpm; lanes 5 and 6, DNA-enzyme complex, 300 cpm. Lanes 1,3, and 5, without enzyme; lanes 2,4, and 6, with enzyme. Lane 7, DNA standards, HinfI digest of pBR322. Sizes are given in nucleotides.

detected in the absence of AdoMet or its analogues (figure 3, lanes 1 and 6). With both these enzymes sinefungin was more effective than AdoMet in stimulating complex formation (figure 3, lanes 3 and 8). With MspI L-S-adenosylhomocysteine stimulated the reaction to a greater extent than D-S-adenosylhomocysteine (figure 3, lane 4 and 5), whereas neither of these compounds stimulated binding of the HpaII methylase (figure 3, lanes 9 and 10).

The effect of these analogues on the reaction of the HhaI methylase differed from these patterns (figure 4). This enzyme,

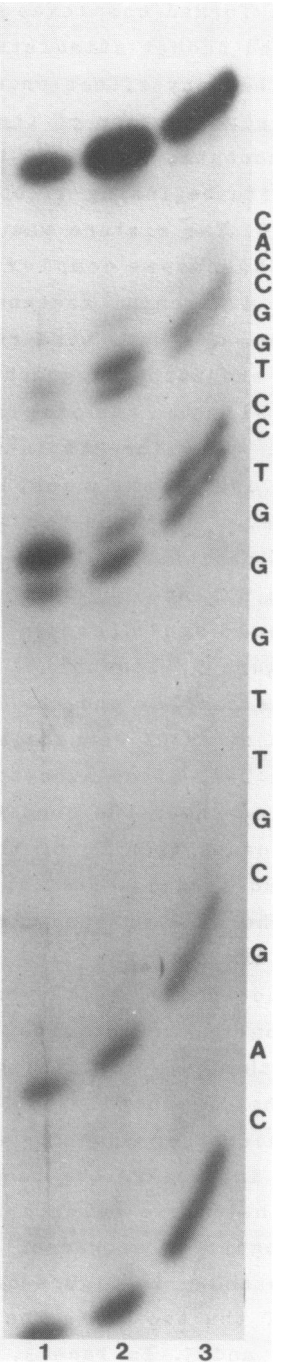
like the EcoRII enzyme, formed complexes with DNA in the absence of AdoMet. Sinefungin and AdoMet stimulated the reaction but none of the other analogues had any effect on complex formation.

Isolation and characterization of the DNA-enzyme complex- EcoRII methylase was incubated with a 32 bp AvaI-HhaI fragment containing one EcoRII site beginning 17 bp from the labeled end as described in figure 5. The mixture was electrophoresed on an 8% gel and the DNA and DNA-enzyme complex eluted from the gel in 0.1% SDS containing 0.5 M ammonium acetate. After concentration by ethanol precipitation, aliquots were reincubated with enzyme and rerun on an 8% gel and autoradiographed.

32,000 cpm were applied to the original gel and 20,000 cpm recovered from the gel, 4,800, or 24% in the complex. Less than 6% of the unreacted DNA could form a complex with fresh enzyme (figure 5, lane 2). However when the isolated DNA-enzyme complex was analyzed 54% of the radioactivity now had the mobility of uncomplexed DNA (figure 5, lane 5). Of the DNA that had dissociated from the complex 44% could again react with fresh enzyme to form a DNA-enzyme complex (figure 5, lane 6).

Both the DNA-enzyme complex and the free DNA were treated with piperidine and analyzed on a 20% sequencing polyacrylamide gel along with the original DNA. Since azacytosine is base labile, treatment with piperidine under the conditions described by Maxam and Gilbert (7) should cause opening of the azacytosine ring followed by elimination and strand scission. Thus bands should appear on the sequencing gel wherever an azacytosine is present in the DNA fragment. Since 12% of the cytosines are substituted by azacytosine a series of bands should appear. If the enzyme binds to azacytosine at a particular position in the sequence than that site should appear more intense in the lane containing the DNA-enzyme complex and depleted in the lane of uncomplexed DNA. Furthermore, cytosines in the sequence other than the one important for binding should be less intense in the lane prepared from the DNA-enzyme complex if the enzyme selects fragments that contain azacytosine in the recognition sequence.

This experiment is shown in figure 6. It is evident that the relative intensities of the two Cs in the sequence CCTGG varies in the three lanes 1, 2 and 3. In lane 3, the lane containing the


CACCGGTCCTGGGGTTTGCGAC
1
2
3

starting material these two Cs are of equal intensity, indicating the equal incorporation of azacytosine in these two positions. However when this DNA is separated into two components on the basis of their reactivity with the EcoRII methylase the relative concentration of azacytosine in these two positions changes. In lane 2, containing the DNA that does not react with the enzyme, the relative intensity of the first C has increased whereas in lane 1, the lane containing DNA that had reacted with the enzyme, it is the relative intensity of the second C in this sequence that has increased indicating that this is the azacytosine that reacts with the enzyme.

DISCUSSION

Azacytidine has been demonstrated to inhibit the formation of 5-methylcytosine in DNA in both procaryotic and eukaryotic cells (8-10). The drug inhibits the DNA(cytosine-5)methyltransferases present in these cells. The cells must be capable of synthesizing DNA in order for this inhibition to occur. It was therefore concluded by several investigators that the drug had to be incorporated into DNA for this inhibition to occur and that it was azacytosine-containing DNA that was the actual enzyme inhibitor (11-13). Further experiments showed that azacytosine-containing DNA is a potent inhibitor of procaryotic DNA(5-cytosine)methyltransferases *in vitro* but does not inhibit the DNA(N^6-adenine)-methyltransferase (1).

If azaC-DNA is an inhibitor of these enzymes than this DNA should form a stable complex with these methylases. Several investigators have now demonstrated such complexes (2, 13-15). The nature and stability of these complexes have differed with different enzymes. Christman et al. (15) reported that the mammalian methylase-azaC-DNA complex could be isolated in the presence of 0.6% Sarkosyl and 0.5 M sodium chloride by a filter binding assay

Figure 6. Sequencing gel analysis of azaC-DNA and the enzyme-azaC-DNA complex following hydrolysis with piperidine. The DNA fragments were the same as those used in figure 5. They were treated with 1 M piperidine at 90° C for 30 min as described in "Methods" prior to electrophoresis on a 20% denaturing polyacrylamide gel (7). Lane 1, azaC-DNA-enzyme complex; lane 2, uncomplexed azaC-DNA; lane 3, azaC-DNA not previously treated with enzyme.

but that the complex was not stable in 0.05% SDS. Binding was not dependent on the presence of AdoMet. Santi et al. (14) studied the procaryotic HpaII methylase, also with a filter binding technique. This methylase-azaC-DNA complex did not undergo detectable dissociation for three days and was stable to treatment with 1% SDS. However AdoMet did not stimulate the reaction and digestion of the DNA with MspI endonuclease, an isoschizomer of HpaII, did not eliminate binding.

Although binding of azaC-DNA fragments to the EcoRII methylase was not reversed by 1% SDS, 6 M urea, nor by precipitation with ethanol, in this study we find that the complex dissociates slowly over a period of days in the presence of 0.1% SDS. A fraction, 44%, of the azaC-DNA liberated during this process can react again with fresh enzyme to form a complex. Heat, 95°C for 15 min, or extraction of the complex with phenol will also cause dissociation of the enzyme-DNA complex (unpublished observations). Although the binding is clearly more stable than that reported for the eukaryotic enzyme it is not irreversible.

The requirement for AdoMet is easily demonstrated for all four enzymes used in this study. No binding could be detected in the case of the MspI and HpaII enzymes in the absence of AdoMet. With the EcoRII and HhaI enzymes binding occurred in its absence but it was stimulated by the addition of AdoMet. In all cases the analogue sinefungin was as, or more effective than AdoMet in stimulating the extent of binding. Methylation of the DNA is therefore not necessary for binding to occur. However from this data we cannot tell if the analogues stimulate binding of the enzyme to DNA, stimulate the rate of formation of a bond to azacytosine in the DNA once binding has occurred, or stimulate movement of the enzyme along the DNA until it interacts with a recognition sequence. Attempts to isolate enzyme-DNA complexes with control DNA fragments were not successful when sinefungin was used in the reaction mixtures. In these experiments the reaction was terminated by addition of E. coli DNA prior to electrophoresis.

The ability of the AdoMet analogues to stimulate formation of the azaC-DNA complex was not related to their affinity for the enzyme as determined by their ability to inhibit the methylation reaction. The analogues sinefungin and L-S-adenosylhomocys-

teine were tested at a concentration 10 and 70 times their respective K_i's and sinefungin was more effective in stimulating complex formation even though it has a greater K_i for the enzyme. The effect is not due to a change in the stability of the enzyme caused by the addition of the analogues since AdoMet did not alter the stability of the enzyme under the conditions of the reaction. The analogues probably cause a conformational change to occur in the enzyme as has been described in the case of the binding of type I enzymes to DNA (16).

There are 2 cytosines in each of the strands of DNA in the sequence that is normally methylated by the EcoRII enzyme. One of these is methylated, in each strand, by the methylase (3). The experiment described in figure 6 was performed to determine which cytosine, when substituted by azacytosine, the enzyme bound to. The fragment used in figure 6 has nine cytosines. By the Poisson distribution 33% of the strands will not contain azacytosine and 31% will contain more than one azacytosine. The fragments would therefore contain both unsubstituted DNA as well as DNA in which a fraction of the cytosines were replaced by 5-azacytosine. The enzyme will bind to fragments having azacytosine in the appropriate position in either strand.

The bound DNA was hydrolyzed with piperidine and the fragments run on a sequencing gel. Of the bound fragments 50% should have azacytosine in the appropriate position in the CCTGG sequence, and 16% of the DNA should remain unhydrolyzed. From the autoradiogram (figure 6,lane 1) it is evident that the relative intensity of the band corresponding to the second cytosine in the CCTGG sequence is increased, whereas in the control lane (lane 3) these bands are of equal intensity. Furthermore, in the uncomplexed DNA, lane 2 of this figure, it is evident that the corresponding band is decreased in intensity, although not absent. Its presence in this lane is due, in part, to the presence of reactive DNA in this fraction. It may also be due to the presence of ring opened forms of azacytosine which would not be expected to react with the enzyme but would be degraded by piperidine. The enzyme therefore must bind to azacytosine in the position that would normally be methylated for a stable DNA-enzyme complex to form.

Walsh (17) and Santi (18), proposed that the enzyme would bind to the 6-position of cytosine in DNA to catalyze the methylation reaction, in analogy with the reaction mechanism of thymidylate synthetase. They proposed that binding to the 6-position of azacytosine would result in a stable bond. Walsh proposed that the enzyme-azacytosine complex might undergo rearrangement to release a formylated enzyme product. The data presented in this paper are consistent with the proposal of an enzyme-azacytosine adduct at the site in DNA that would normally be methylated. After denaturation of the enzyme with SDS dissociation of the complex, leaving the azacytosine intact, appears to occur more rapidly than other forms of rearrangement.

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