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Detection of a CpA methylase in an insect system: characterization and substrate specificity

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Abstract

A cytosine-specific DNA methyltransferase (EC 2.1.1.37) has been purified to near homogeneity from a mealybug (*Planococcus lilacinus*). The enzyme can methylate cytosine residues in CpG sequences as well as CpA sequences. The apparent molecular weight of the enzyme was estimated as 135,000 daltons by FPLC. The enzyme exhibits a processive mode of action and a salt dependance similar to mammalian methylases. Mealybug methylase exhibits a preference for denatured DNA substrates. (Mol Cell Biochem **110:** 103–111, 1992)

Key words: DNA methylation, coccids, processive enzyme, RNA inhibition, CpI methylation

Introduction

The importance of DNA methylation in gene expression is becoming increasingly evident [1–3]. In eukaryotes most of the cytosines methylated *in vivo* are found in the dinucleotide CpG [4, 5]. In most insects the content of 5-methyl cytosine (5mC) in DNA is very low [6, 7]. However, in *Planococcus lilacinus* (Coccidae; Homoptera; Insecta) significant levels of DNA methylation have been reported [8, 9]. Additionally, nearest neighbour analysis of DNA from *P. lilacinus* suggested the presence of 5mC in dinucleotides other than CpG [8]. *Planococcus lilacinus* exhibits sex-specific differential regulation of homologous chromosomes [10]. During early embryonic development, the paternal set of chromosomes is inactivated in certain embryos which develop into males, whereas embryos with two functional sets of chromosomes develop as females. Thus the paternal chromosomes in this system are said to remember their parental origin. DNA methylation is believed to be a basis for this phenomenone which is referred to as chromosome imprinting [11, 12]. Several models involving DNA methylation have been proposed to explain chromosome imprinting and inactivation in mealybugs [13, 14].

S-adenosyl methionine-DNA-methyltransferases (EC 2.1.1.37, DNA methylases), catalyse the transfer of methyl group from S-adenosyl methionine onto cyto-

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sine or adenine residues in DNA. DNA methylases have been purified from a variety of eukaryotic sources including rat liver [15], HeLa cells [16] and human placenta [17]. *In vitro,* all of the DNA methylases have shown specificity for CpG dinucleotide [5, 18, 15, 19]. On the other hand the DNA methylase from mealybugs can methylate not only CpG but also CpA dinucleotides. This property is interesting in the light ofthe previous observation [8] that mealybug genomic DNA has 5mC in sequences other than CpG dinucleotides. The mealybugs exhibiting a selective nonrandom inactivation of chromosomes provide a unique opportunity to study the correlation between DNA methylation, imprinting and chromatin condensation, if any. Here we report the detection, purification and characterization of a DNA methylase from a mealybug. The results reported here suggest that there might be a single enzyme mediating both CpG as well as CpA methylation. However the enzyme did not accept poly pyrimidines as substrates.

Materials and methods

Stock cultures of a mealybug, provisionally identified as *Planococcus lilacinus* were obtained from the horticultural research station, Coorg, India. They were maintained on pumpkins at room temperature. *E. coli* mutant strain [dcm$^-$ dam$^-$] was a gift from Dr. V. Nagaraja. Synthetic polymers were obtained from Pharmacia United, Bangalore. ^{14}C-methyl S-adenosyl methionine (specific activity 62 μCi/μmol), was a gift from Dr. C. Vijayasarathy. Most of the DNA polymers and other chemicals were obtained from Sigma Chemical Company, St. Louis USA, and Boehringer Manheim.

Preparation of nuclei

Gravid female insects (which would be expected to contain embryos of both sexes) were used for preparing nuclei. The insects were washed briefly in cold acetone to remove the wax coating and were suspended in a buffer containing 10 mM Tris HCl pH 7.4, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol and 10% glycerol and homogenised with a teflon homogeniser. After a brief centrifugation at 3000 rpm, the nuclei were suspended in 0.25 M sucrose and were purified sequentially over sucrose cushions containing 0.88 M and 1.1 M sucrose.

***In vitro** assay for methylase*

The reaction was carried out according to Simon *et al.* [15] in a total volume of 50 μl. About 180 μg of protein, 48 μM of ^{14}C-methyl S-adomet and 12 μg of DNA were used in each assay. KCl at 50 mM or 150 mM was added to the assay mixture for native and denatured DNA substrates respectively. The reaction was carried out at 37° C for 30 min and monitored as described before [20].

Immunochemical assay for detection of 5mC in DNA was carried out according to Achwal and Chandra [21]. After the *in vitro* reaction, product DNA was reextracted with phenol-chloroform and precipitated with ethanol. Immunochemical reactions using antibodies directed against 5mC or 6mA were carried out on these DNA samples, dissolved in 10 mM Tris-HCl, and 1 mM EDTA pH 7.4. The DNA samples were spotted on nitrocellulose filters, fixed by baking in a vacuum oven and treated with antibodies [21]. The antibodies were raised in rabbits using BSA conjugates oof 5-methylcytidine or 6-methyladenine as antigens [21].

Preparation of the enzyme

Crude nuclear extracts were prepared by the method of Simon *et al.* [15]. After 0.8 M KCl extraction, the proteins were dialysed against buffer A containing 10 mM Tris HCl, pH7.4, 2 mM EDTA, 20 mM 2-mercaptoethanol, 1 mM PMSF and 10% glycerol. The dialysate was loaded on a DEAE cellulose column (bed volume 40–45 ml), the proteins were eluted with a linear gradient of 0–0.4 M KCl in buffer A. The peak fractions showing methylase activity were pooled, dialysed and loaded on a hydroxy-apatite column (bed volume 3–4 ml) and eluted with a step gradient of phosphate. In the final step of purification, the active fractions from the hydroxyapatite column were loaded on a SAH-agarose column which was prepared by the method of Humayun and Jacob [22]. The purified enzyme fraction was tested for the presence of nuclease activity by incubating with supercoiled plasmid DNA and analysing the DNA by agarose gel electrophoresis subsequently.

Table 1. Detection of DNA methylase activity in cell free extracts from mealybug

Substrate DNA	Specific activity (pmoles/mg/hr)
dcm$^-$ dam$^-$ *E. coli*	0.438
E. coli B	0.39
poly(dG-m^{5}dC)·poly(dG-m^{5}dC)	0
poly(dG-dC)·poly(dG-dC)	0.79
Yeast total RNA	0
Rat liver tRNA	0

The protein fraction from affinity column was labelled with I^{125} [23] and electrophoresis was carried out according to Laemmli [24]. The gel was dried and subjected to autoradiography. Protein concentration was estimated by the method of Bradford [25]. DNA was denatured by boiling in 0.15 M NaCl at 100° C for 10 min and cooled rapidly on ice. Single stranded DNA was separated from double stranded DNA by using a hydroxyapatite column [26].

The molecular weight of the enzyme was determined by FPLC using a Superose-6 column, equilibrated with 100 mM Tris HCl pH 8.0, 1 mM EDTA, 20 mM 2-mercaptoethanol and 10% glycerol. The protein fraction was assayed for methylase activity and the molecular weight was calculated using a standard curve drawn from elution profile of standards like Alkaline phosphatase (140 kd), Bovine serum albumin dimer (132 kd), BSA monomer (66 kd), ovalbumin (45 kd) and chymotrypsinogen (25 kd).

Results

Purification of the enzyme

Protein extracts were prepared from nuclei purified from gravid females by the procedures described under methods and the presence of DNA methylase in these extracts was detected using undermethylated DNA from *E. coli* B, dcm⁻ dam⁻ *E. coli,* and synthetic polymers (Table 1). Undermethylated DNAs and poly (dG-dC)·poly(dG-dC) were efficiently methylated, whereas methylated poly(dG-d5mC)·poly(dG-d5mC) and RNA samples were not methylated. To confirm the formation of 5mC *in vitro,* the substrate DNA was reisolated from the *in vitro* reaction mixture, spotted on nitrocellulose filter and subjected to immunochemical reaction using antibodies directed against 5mC. As seen in Fig. 1, 5mC could be detected only in DNA methylated *in vitro*. The products of *in vitro* methylation

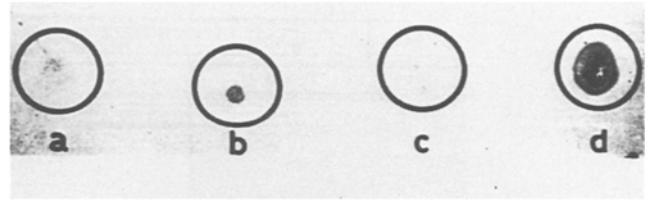


Fig. 1. Detection of 5mC in DNA using antibodies directed against 5mC and avidin-biotinylated peroxidase detection system. 500 ng of DNA from: (a) λ phage (unmethylated) (b) phage λ (methylated) and approximately 1.0–BDg of DNA from (c) dcm⁻ dam⁻ *E. coli* (d) dcm⁻ dam⁻ *E. coli* methylated *in vitro* with mealybug DNA methylase, were immobilised on nitrocellulose paper and subjected to immunochemical reaction.

failed to show any reaction with antibodies directed against 6mA.

Using DNA from dcm⁻ dam⁻ *E. coli* as substrate, the methylase from *P. lilacinus* was purified through DEAE-cellulose and hydroxyapatite column chromatography, on which the enzyme yielded a single activity peak at 0.16 M KCl and 0.3 M phosphate respectively. The methylase was then subjected to affinity chromatography on S-adenosyl homocysteine agarose column and eluted with 0.3 M KCl. The purification achieved at different steps is indicated in Table 2. Purification greater than 500-fold was achieved after affinity chromatography. The enzyme gave a single band on electrophoresis in a 7.5% polyacrylamide gel (Fig. 2). Using fast protein liquid chromatography (FPLC) the molecular weight of the enzyme was calculated as 135 kilodaltons. The purified enzyme was used in all further characterizations.

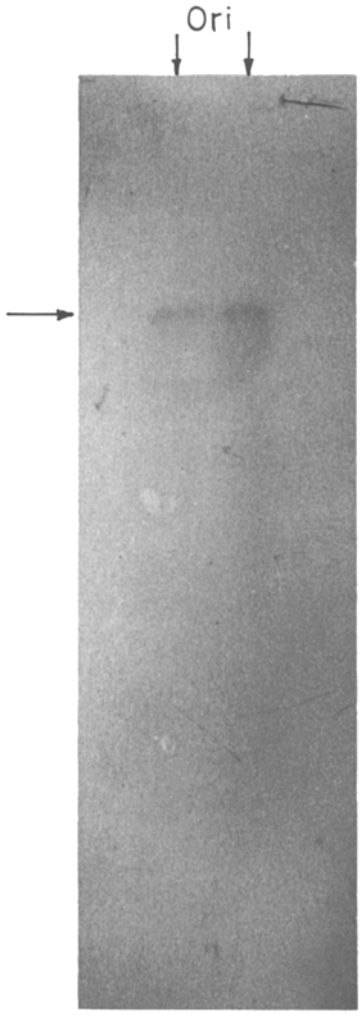
Substrate specificity

In order to study substrate specificity, various synthetic polymers were used as substrates (Table 3). It was observed that the enzyme prefers only alternating purine-pyrimidine sequences. It was also noted that the

Table 2. Purification of DNA cytosine 5-methyltransferase from *Planococcus lilacinus*

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification (fold)
Crude extract	100	43.80	0.438	—
DEAE-cellulose	5.5	74.20	13.5	31
Hydroxyapatite	0.210	12.18	58.0	132
S-adenosyl homocysteine-agarose	0.053	12.60	238.0	543

One unit of activity is defined as the amount of enzyme that catalyses the incorporation of one picomole of methyl groups into DNA from dcm⁻ dam⁻ *E. coli* in sixty minutes at 37° C under standard assay conditions.



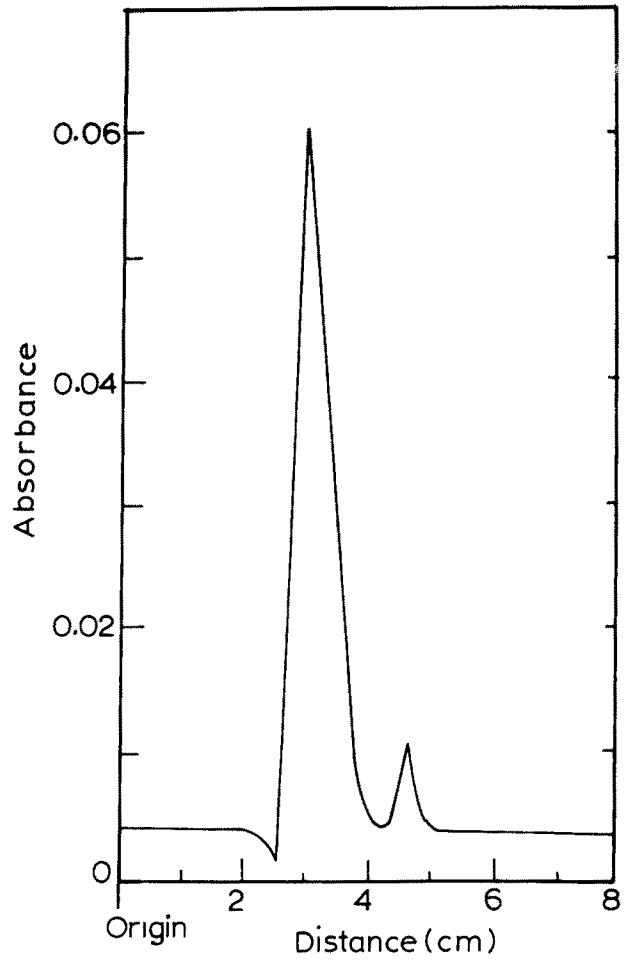


Fig. 2. Electrophoresis of the purified enzyme on non-denaturing polyacrylamide gel (7.5%). The protein fraction from SAH-agarose affinity column was iodinated and separated on the gel.
(a) The autoradiogram of the dried gel is shown here. The two lanes represent two different concentrations of the same fraction. The arrow indicates the position of the only protein band seen indicating near homogeneity of the enzyme.
(b) Densitometeric tracing of the negative. Absorbance is expressed in arbitreary units.

enzyme can methylate both CpG as well as CpA sequences. The kinetics of the reaction with various synthetic substrates show that apart from poly(dG-dC)·poly(dG-dC) both poly(dA-dC)·poly(dT-dG) and poly(dI-dC)·poly(dI-dC) are methylated (Fig. 3). This activity correlates well with the presence of 5mC in both CpG and CpA sequences in the genomic DNA of mealybugs [8]. Thus the DNA methylase from *P. lilacinus* accepts completely unmethylated DNA as substrate thereby indicating the presence of *de novo* methylase activity.

Enzyme assays were carried out with double- as well

Table 3. Substrate specificity of the enzyme

Synthetic polymer	Enzyme activity
poly(dG-dC)·poly(dG-dC)	2.1
poly(dA-dC)·poly(dT-dG)	2.0
poly(dA-dG)·poly(dC-dT)	0
poly(dI-dC)·poly(dI-dC)	3.2
poly(dG)·poly(dC)	0
poly(dA)·poly(dT)	0
poly(dI)·poly(dC)	0

Enzyme activity is expressed as pmols of $-CH_3$ group incorporated per μg of polymer in one hour. 180 ng of purified enzyme was used in each assay.

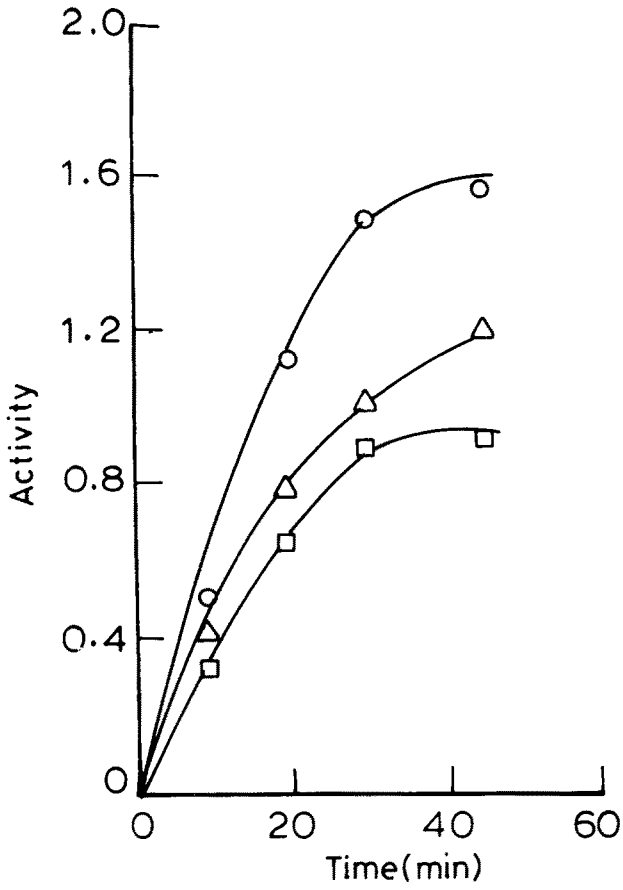


Fig. 3. Substrate specificity of DNA methylase. The purified enzyme was used to monitor the kinetics of methylation of various substrates (○—○—○) poly(dI-dC)·poly(dI-dC); (△—△—△) poly(dA-dC)·poly(dT-dG); (□—□—□) poly(dG-dC)·poly(dG-dC). The activity represents pmols of [^{14}C] methyl group incorporated per μg of the polymer.

Table 4. Methylation of denatured and native forms of DNA

Substrate DNA	Enzyme activity	
	Native	Denatured
E. coli dcm$^-$ dam$^-$	1.15	2.08
E. coli B	1.05	1.8
Micrococcus luteus	1.05	2.27
Saccharomyces cerevisiae	1.02	2.16
Calf thymus	0.19	0.42
Planococcus lilacinus	0.20	0.38
Salmon sperm	0.29	0.48
Rat liver	0.34	0.50

Enzyme activity is expressed as pmol of CH_3-group incorporated per μg of DNA in one hour. 180 ng of purified enzyme was used in each case.

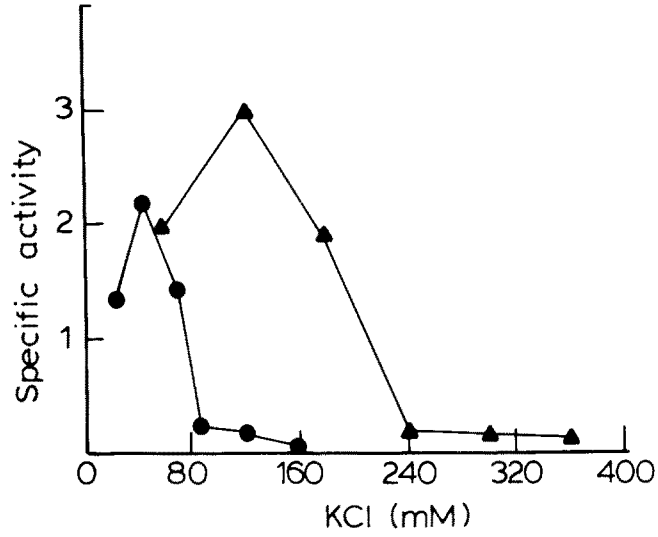


Fig. 4. Effect of salt on methylation. (●—●—●) methylation of native DNA, (▲—▲—▲) methylation of denatured DNA. Equal amounts of DNA (12 μg) was subjected to methylation at varying concentrations of salt. The specific activity represents the methyl group incorporation in pmol/μg protein/hour.

as single-stranded DNA from various sources. As seen in Table 4, the DNA methylase from mealybugs can methylate DNA from several sources that are known to have low levels of modification. DNAs from calf thymus, salmon sperm and rat liver are low methyl acceptors than DNA from dcm$^-$ dam$^-$ *E. coli* and *Micrococcus leuteus*. Denatured DNA was a better substrate than native DNA in all cases tested. The reaction with native DNA was sensitive to salt concentration, and was optimal at 50 mM KCl, but decreased with increasing salt concentration. However, the reaction with denatured DNA was not sensitive to salt up to 150 mM KCl; (Fig. 4). The enzyme seems to form a salt resistant complex with substrate DNA and S-adenosyl methionine at 37° C and therefore addition of upto 200 mM KCl subsequently does not affect the activity even with double stranded DNA (Table 5). In these assays, the enzyme was preincubated with different components of the assay mixture at 37° C and the remaining components of the reaction were added subsequently (Table 5). This indicates that formation of the enzyme-DNA complex is salt sensitive but an effective complex, once formed, is resistant to high salt concentration and can methylate even double stranded DNA.

In order to investigate whether two distinct activities, one for denatured and one for native DNA are present, mixed incubations were carried out. Denatured and native DNA was coincubated at a total concentration of

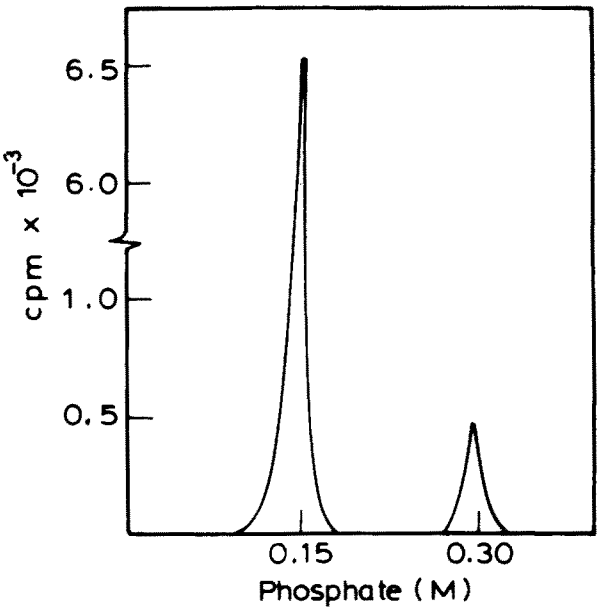


Fig. 5. Separation of products of the reaction with a mixture of native and denatured DNA, on hydroxyapatite; I-denatured DNA II native DNA. The elution was monitored in terms of radioactivity recovered from the column, representing CH_3-group incorporated.

15 μg (7.5 μg each) and 30 μg (15 μg each). At both concentrations the activity observed was not additive but was equivalent to that observed with denatured DNA alone (data not shown). Subsequently when the product DNAs were separated on hydroxyapatite column, almost all the radioactivity was confined to the denatured DNA peak (Fig. 5). These results suggest that both maintenance as well as *de novo* methylation may be carried out by the same enzyme. They also suggest that the methylase probably has a higher affinity for denatured DNA, as denatured DNA is preferentially methylated in assays in which both native and denatured DNA are present.

Mode of action

Two alternative mechanisms are envisaged for the methylation reaction: (i) the enzyme binds to a particular site on the DNA, methylates it, and dissociates from the DNA. This is referred to as the 'rebinding' mode of action: (ii) the enzyme binds to DNA and travels along the length of the molecule, methylating only specific sites. This is referred to as the 'processive' mode of action [27]. A distinction between these two modes of action can be made by sequential addition of substrates differing in their methyl acceptor capacity [28]. In this study, the reaction was initiated with a DNA sample as the first substrate and after five minutes of incubation at

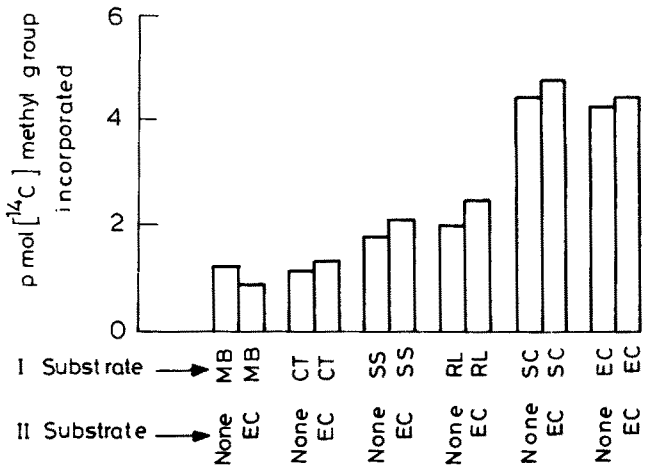


Fig. 6. Mode of action of mealybug DNA methylase. Reaction with two substrates, added sequentially (as described in the text).

37° C, the second substrate was added. DNA samples (15 μg) from dcm⁻ dam⁻ *E. coli,* yeast, calf thymus, rat liver, salmon sperm and mealybug were used as the first substrate in different experiments. In each case DNA from dcm⁻ dam⁻ *E. coli* (15 μg) was used as the second substrate and the reaction was continued for 30 min after addition of the second substrate. The results are shown in Fig. 6. It is seen that methylation remains at a level characteristic of the first substrate in every case. For instance, DNA from calf thymus is a low-methyl-acceptor whereas DNA from dcm⁻ dam⁻ *E. coli* is a high methyl-acceptor. When DNA from calf thymus is used as the first substrate and DNA from dcm⁻ dam⁻ *E. coli* as the second substrate, the levels of methylation after 30 min of incubation corresponds to that of calf thymus DNA alone. This suggests that once bound to

Table 5. Formation of salt resistant complex by the enzyme

A (0° C)	B (37° C)	Specific Activity
Enzyme + DNA + S-adomet	None	1.42
Enzyme + DNA + KCl + S-adomet	None	0
Enzyme + DNA + S-adomet	KCl	2.5
Enzyme + DNA	S-adomet + KCl	0.63
Enzyme	KCl + DNA + S-adomet	0

Components under column A were added at 0° C, preincubated at 37° C for 5 min before addition of components under column B. Reaction was continued for 30 min at 37° C. Specific activity is expressed as pmol CH_3/μg protein/hour. KCl was added to a final concentration of 200 mM.

the substrate DNA, the enzyme traverses along the length of the molecule and does not dissociate to methylate the second substrate. These results suggest that the mealybug DNA methylase follows a processive mode of action.

Effect of RNA on enzyme activity

Mealybug methylase was found to be specific to DNA and no activity was observed when RNA was used as the substrate (Table 1). However, when DNA was used along with RNA at lower concentrations (2 μg of RNA and 12 μg DNA) only 25% of the expected activity was observed. Almost 100% inhibition was seen at 10 μg of RNA (Fig. 7). Further, when RNA treated with RNase A is used in the reaction, no inhibition is seen (Fig. 7). These results indicate that ribopolymers may act as inhibitors of mealybug DNA methylase but not ribooligomers. In mixed incubations, when RNA was used as the first substrate and after 5 min of incubation at 37° C, DNA from dcm$^-$ dam$^-$ *E. coli* was added and the reaction continued for 30 min, there was no detectable activity. However, in a similar assay system when DNA was used as the first substrate and RNA as the second substrate, normal levels of activity was detected and no inhibition was observed (data not shown). Therefore it appears that inhibition is caused by binding of RNA to the enzyme.

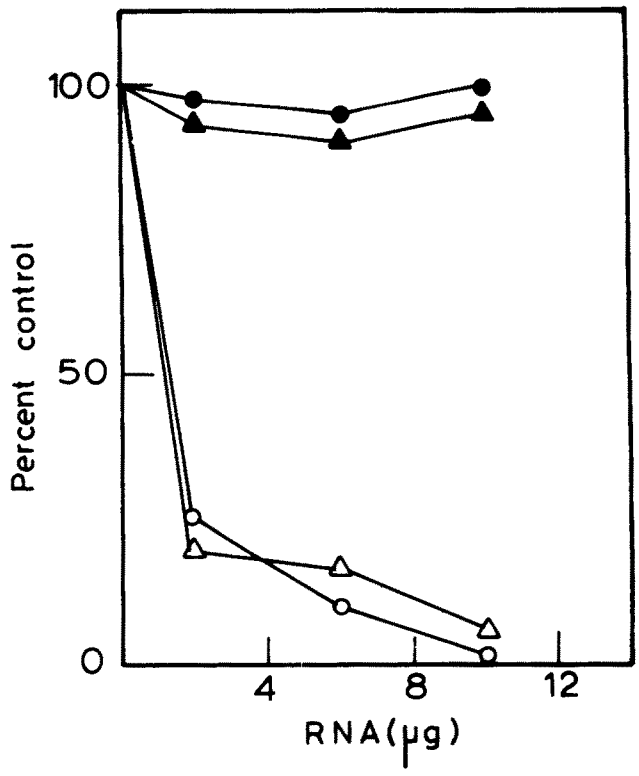


Fig. 7. Inhibition of methylation by RNA. (○—○—○) Yeast total RNA, (△—△—△) Rat liver tRNA, (●—●—●) Yeast total RNA treated with RNase A, (▲—▲—▲) Rat liver tRNA pretreated with RNase A. Equal amounts (12 μg) of DNA from dcm$^-$ dam$^-$ *E. coli* was used in all the assays. The activity is expressed as a percentage of the activity obtained with the same amount (12 μg) of DNA in absence of RNA.

Discussion

The DNA methylase from mealybug methylates only cytosine residues in DNA. The enzyme can methylate both denatured as well as native DNA substrates. This property is seen in DNA methylases from several mammalian sources [5, 17]. The DNA methylase from mealybug shows preferential methylation of denatured DNA even under low salt (50 mM) conditions. This is not a universal property of DNA methylases, but Adams *et al.* [28] have reported a similar phenomenon with DNA methylase purified from mouse ascites cells. The sequence specificity of the methylase as assessed by using synthetic polymers shows that both CpG as well as CpA dinucleotides are methylated by this enzyme. This property has so far not been demonstrated for any of the purified DNA methylases with synthetic polymers as substrate. However 5mC was detected in CpA dinucleotides at low frequency in retroviral genomes methylated by DNA methylase from regenerating rat liver [29]. But the same enzyme was reported to have no activity with the alternating polymer poly(dA-dC)·poly(dT-dG) *in vitro* [15]. The ability of mealybug methylase to methylate CpA sequences correlates well with previous results which indicated the presence of 5mC in mealybug genomic DNA in dinucleotides other than CpG [8]. The activity of DNA methylase varies during different stages of development of the mealybug, however, the ability to methylate CpA sequences is detectable at all stages at which CpG methylation is detected [20]. Therefore it appears that CpA methylation may be an important post-replicative modification of DNA in this system. Poly(dI-dC)·poly(dI-dC) shows a higher levle of methyl accepting capacity than poly(dG-dC)·poly (dG-dC). Inosine containing polymers have been shown to be better substrates for metylases from human as well as mouse cells [30, 19].

The mode of action of DNA methylases has previously been studied in case of enzyme isolated from rat liver [27, 31, 15] and human placenta [17]. Enzymes from both these sources are known to exhibit a processive

mode of action. However, a processive mode of action could not be confirmed for metylase from Kreb II ascites cells [32]. The results reported here indicate that the mealybug DNA methylase also exhibits a processive mode of action. We have demonstrated that the mealybug enzyme forms a salt-resistant complex at 37° C, thus supporting the hypothesis that there might be two distinct steps in its activity: one a salt-sensitive binding of the enzyme to the substrate DNA and another a salt-resistant step leading to the methylation of cytosine residues. The mealybug DNA methylase is inhibited by competitors for S-adomet like SAH and SIBA (data not shown). It is also inhibited by RNA. On coincubation with RNA, a severe inhibition of the reaction was observed, but once the enzyme is bound to DNA, no inhibition is observed with RNA. The inhibition of DNA methylation by RNA has also been demonstrated in the case of HeLa cell DNA methylase [33]. These observations suggest that RNA probably can compete with DNA for binding to the enzyme. This interaction may be relevant for regulation of methylation *in vivo*. In view of the presence of RNA in DNA replication complexes, the inhibition of DNA methylation by RNA may have a role in bringing about demethylation, by inhibiting methylation of hemimethylated DNA in the replication fork [34].

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