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Single-stranded DNA binding and methylation by EcoP1I DNA methyltransferase☆

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Abstract

EcoP1I methyltransferase (M.EcoP1I) belongs to the type III restriction–modification system encoded by prophage P1 that infects *Escherichia coli*. Binding of M.EcoP1I to double-stranded DNA and single-stranded DNA has been characterized. Binding to both single- and double-stranded DNA could be competed out by unlabeled single-stranded DNA. Metal ions did not influence DNA binding. Interestingly, M.EcoP1I was able to methylate single-stranded DNA. Kinetic parameters were determined for single- and double-stranded DNA methylation. This feature of the enzyme probably functions in protecting the phage genome from restriction by type III restriction enzymes and thus could be considered as an anti-restriction system. This study describing in vitro methylation of single-stranded DNA by the type III methyltransferase EcoP1I allows understanding of the mechanism of action of these enzymes and also their role in the biology of single-stranded phages.

Keywords: Anti-restriction; Binding; Methylation; single-stranded DNA

Double-stranded DNA is the substrate for DNA methyltransferases. However, some methyltransferases such as EcoKDam methyltransferase (Dam MTase) [1] and others that are a part of R–M systems such as BcnI [2] and DpnII [3] are capable of methylating single-stranded DNA also. These R–M systems have one of the two MTases of the same specificity methylating single-stranded DNA. *Escherichia coli* Dam MTase performs numerous functions in the cell. It participates in DNA strand discrimination during post-replicative mismatch repair, phage protection, gene regulation, and control of DNA replication [4]. M.EcoKDam is active on denatured DNA and single-stranded synthetic oligonucleotides containing GATC sites. Upon interaction with single-stranded oligonucleotides, the enzyme forms a duplex structure within the GATC site, which is the substrate for methylation [1]. This may reflect a functional role for the enzyme in the cell.

DNA methyltransferases of some *Haemophilus* strains are able to methylate denatured and single-stranded DNA. A mutant methyltransferase of HinfI with carboxyl-terminal 97 amino acids deleted, encodes both recognition and catalytic functions and was found to preferentially methylate single-stranded DNA [5]. The DpnII restriction system found in a strain of *Streptococcus pneumoniae* contains three genes, *dpnM*, *dpnA*, and *dpnB* that encode two DNA methyltransferases, DpnM, DpnA and an endonuclease, DpnII, respectively. DpnA preferentially methylates single-stranded DNA and also sequences other than 5′-GAT C-3′, when the altered base is guanine. Single-strand-specific MTase may be required to enhance plasmid transmission to the cells during transformation, which is known to proceed via a single-stranded intermediate [2].

Sequence analysis of the BcnI R–M system revealed the presence of a functional open reading frame encoding a second cytosine-*N*4-methyltransferase, M.*Bcn*IA along with the genes for R.BcnI and M.BcnIB [6]. M.BcnIA showed some activity towards non-canonical sequences and was equally efficient in

☆ *Abbreviations:* AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; ds, double-stranded; Mod, modification subunit; R–M, restriction–modification; Res, restriction subunit; sf, sinefungin; ss, single-stranded.

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methylating single-stranded and double-stranded DNA. It has been proposed that during plasmid transmission, methylation of the incoming strand by M.BcnIA would protect the subsequently reconstituted plasmid from R.BcnI cleavage [2]. The CpG methylase, M.SssI (from *Spiroplasma* species Strain MQ1), methylates the sequence 5′-CG-3′ at C5 position both in the double- and single-stranded context [7]. Murine DNA (cytosine-5)-methyltransferase (Dnmt1) is capable of methylating single-stranded DNA in addition to double-stranded DNA [8]. Presence of a proximal 5mC increased single-stranded DNA methylation upto 50-fold [9].

EcoP1I methyltransferase is a member of the type III R–M system. The restriction enzyme (containing both the Res and Mod subunits) is a very efficient methylase. The modification enzyme (M.EcoP1I) made up of Mod subunits recognizes the asymmetric sequence 5′-AGACC-3′ and methylates the internal adenine. One peculiar feature of methylation by type III R–M systems is that only one strand of the asymmetric recognition sequence is methylated [10].

Despite intensive studies of the type III R–M systems they have not been fully characterized from a biochemical point of view. In this study we have investigated single-strand DNA binding and methylation by the EcoP1I methyltransferase. This is the first time that such an activity has been reported among the type III R–M systems. The functional significance of this unique feature of the enzyme is discussed.

Materials and methods

Bacterial strains and plasmid vectors. Escherichia coli strain JM109 was used as a host for overexpression and purification of EcoP1I DNA methytransferase. M.EcoP1I was purified from the construct pVK1, which harbours the SmaI–HindIII fragment of BamHI-4 fragment of P1 genome [11]. pUC18, pUC19, M13mp18, and M13 phage DNA were isolated as described [12].

Chemicals. S-Adenosyl-L-methionine (AdoMet), sinefungin (sf), *S*-adenosyl-L-homocysteine (AdoHcy), adenosine-5′-triphosphate (sodium salt), ampicillin, Hepes, polyethyleneimine (PEI), Coomassie brilliant blue R-250, and RNase A were from Sigma Chemical Company, USA. Restriction and modification enzymes were obtained from New England Biolabs, USA. [γ^{32}-ATP] and [^{3}H]-AdoMet were purchased from NEN, USA. Centricon-30 was purchased from Amicon, USA.

General recombinant techniques. Restriction enzymes, Klenow fragment of DNA polymerase I, and T_4 polynucleotide kinase were used according to manufacturers' recommendations. Digestion with type II restriction enzymes and DNA electrophoresis were done as described [12]. pUC18 DNA used for methylation assays was prepared by the alkaline lysis method with modifications as described by Sambrook et al. [12]. M13 single-stranded DNA and double-stranded DNA were prepared [12] and concentrations were estimated spectrophotometrically.

Protein purification. Escherichia coli JM109 cells were transformed with the construct pVK1 and used for the expression and purification of EcoP1I DNA methyltransferase as described by Hornby et al. [13] with modifications. Fractions containing enzyme from the heparin–Sepharose chromatography step were dialyzed against buffer A [10 mm Tris–HCl, pH 8.0, 7 mM of 2-mercaptoethanol, 100 mM NaCl, and 10% (v/v) glycerol]. The dialyzed protein was loaded onto a gel filtration column (Superdex-200 FPLC) and eluted using the same buffer. Fractions containing enzyme were pooled and concentrated using a Centricon-30 micro concentrator and was stable up to 2 months. EcoP1I restriction enzyme was purified following the procedure described by Saha and Rao [14]. Protein estimation was carried out as described [15].

Oligonucleotides. The following single- and double-stranded oligonucleotides were used in electrophoretic mobility shift assays

Oligonucleotide 1:
5′-TGAAAGAGGACAGATGAACGGTGTACAGACCAGGC GCATAGGCT GGCTGACCTTCATCAAGAGTAATCTTGA-3′

Oligonucleotide 2:
3′-TTCTCCTGTCTACTTGCCACATGTCTGGTCCGCGTAT CCGACCGACTGGA AGTAGTTCT CATTAGAACT-5′
(complementary to Oligonucleotide 1)

Oligonucleotide S1:
5′-TAGGTCAGAATTCAGCAGACCTAAGTAGCC-3′

Oligonucleotide S2:
5′-ACTTAGGGTCTGCTGAATTCTGACCTA-3′
(complementary to S1)

The underlined region of the oligonucleotide represents the *Eco*P1I recognition sequence

Oligonucleotide S3:
5′-TAGGTCAGAATTCAGCAGTCCCTACGTAGCC-3′
(carries AGTCC instead of AGACC in the underlined region)

Oligonucleotide S4:
5′-GGCTACGTAGGGACTGCTGAATTCTGACCTA-3′
(complementary to S3)

Basic procedures for labeling of oligonucleotides have been described [16].

Electrophoretic mobility shift assay. DNA binding assays were performed as described [16]. Annealing oligonucleotides 1 and 2 formed duplex A. The bands were visualized by autoradiography and quantitated using a Fuji BAS-2000 Phosphor-Imager. Dissociation constant (K_d) determination was based on the radioactivity present in the free DNA. This is more accurate than evaluation based on the bound DNA due to protein–DNA dissociation during electrophoresis. The percentage DNA remaining was plotted against the enzyme concentration and the apparent K_d was determined as enzyme concentration at which half the DNA was free.

Methylation assay. Methylation activity was monitored by the incorporation of tritiated methyl groups into DNA, as described [17].

Results and discussion

DNA binding

Electrophoretic mobility shift assays have revealed interesting information about DNA binding by MTases of all the three classes. The strict requirement for AdoMet in the formation of an enzyme–DNA complex was demonstrated in the case of EcoKI MTase, a member of the type I R–M system [18]. The type II cytosine MTases, M.HhaI and M.HpaII, were found to bind DNA bearing single base mismatches within their recognition sequences [19]. The interaction of the type III R–M enzyme, M.EcoPI5I with DNA revealed that binding constant was in the micromolar range [20]. M.EcoPI5I binds to DNA containing mismatches more tightly than to its cognate sequence, both in the binary

and ternary complex. A simple interpretation of these studies that has been postulated is that destabilizing the base-pair facilitates base flipping and leads to a lower K_d (tighter binding) [21].

Interaction of M.EcoP1I with double-stranded DNA

The extent of binding of *Eco*P1I MTase to DNA was studied using gel mobility shift assays with an end labeled duplex A (see Materials and methods) that carries the EcoP1I recognition sequence. Incubation with DNA in the presence of increasing concentrations of protein resulted in increased binding with a corresponding decrease in the amount of free oligonucleotide (Fig. 1A). The apparent dissociation constant, K_d, was determined (as described in Materials and methods) to be 1.05 μM. It should be noted that as gel shift assay is not an equilibrium method, true K_d values cannot be determined by this procedure. However, this complex was shown to be specific, in that it was not affected by the presence of poly(dI–dC) but was competed by increasing concentrations of the unlabeled oligonucleotide (data not shown). Further, the enzyme was able to bind DNA in the absence of cofactor, AdoMet (data not shown). This is in contrast to DNA binding by the type I enzymes, where bound AdoMet is a prerequisite for DNA binding [18]. In the case of EcoKI MTase, it has been demonstrated that AdoMet binding alters the contact points on DNA [22]. However, in the kinetic mechanism proposed for EcoPI5I DNA MTase, a member of the type III R–M systems [23], it has been postulated that the order of binding of substrates is random. The results obtained with EcoP1I DNA MTase are consistent with this scheme.

Metal ions have been shown to affect the DNA binding property of restriction enzymes and play a role in catalysis [24]. While the role of metal ions in DNA cleavage is well documented, there have been no reports regarding the requirement of metal ions in methylation. Recently, Marks et al. [25] reported the requirement of magnesium for activity by AhdI DNA methyltransferase, while others such as M.Eco57I [26], BcgI [27], and AloI [28] are stimulated by metal ions. Binding of EcoP15I DNA methyltransferase (a member of the type III R–M enzymes) to its recognition sequence was stronger in the presence of Mn^{2+} than in the presence of Mg^{2+}, although Mg^{2+} is the metal ion of choice for enzyme activity [20]. DNA binding was seen in the presence and absence of metal ions (Fig. 1B). In the presence of Co^{2+} and Fe^{2+}, maximal binding was seen. In the presence of Fe^{2+}, a complex of lower mobility was seen in the well (Fig. 1B, lane 8). The nature of this lower mobility complex remains unknown. It is possible that the presence of metal ions could result in precipitation or aggregation of DNA in the absence of protein.

Interaction of M.EcoP1I with single-stranded DNA

Interestingly, EcoP1I MTase was found to bind ssDNA. To investigate the ability of the enzyme to bind both or only one of the two DNA strands in the recognition sequence, four different end-labeled 31 mer substrates were used. Mobility shift assays using these four substrates revealed that the enzyme does bind all four ssDNA oligonucleotides (Fig. 2A). However, binding to S1 oligonucleotide bearing the recognition sequence is appreciably more than the binding to either

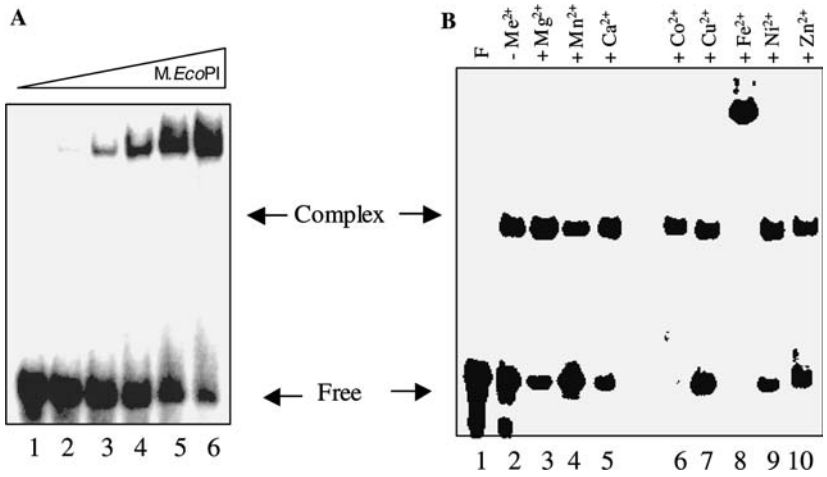

Fig. 1. Binding of EcoP1I DNA MTase to DNA. (A) The 5′-end-labeled duplex A (approximately 100 nM) and increasing amounts of EcoP1I DNA MTase were incubated in binding buffer for 10 min on ice and analyzed on a 6% polyacrylamide gel as described in Materials and methods. Lane 1, no protein; lane 2, 0.068 μM; lane 3, 0.136 μM; lane 4, 0.34 μM; lane 5, 0.68 μM; and lane 6, 1.36 μM. (B) EcoP1I DNA MTase binding to double-stranded DNA in the presence of divalent metal ions. The 5′-end-labeled duplex A (approximately 100 nM) was incubated with EcoP1I DNA MTase (1.36 μM) and 10 mM Me^{2+}, in binding buffer on ice for 10 min and analyzed as described in Materials and methods. Lane 1, no protein; lane 2, no metal ion; lane 3, Mg^{2+}; lane 4, Mn^{2+}; lane 5, Ca^{2+}; lane 6, Co^{2+}; lane 7, Cu^{2+}; lane 8, Fe^{2+}; lane 9, Ni^{2+}; and lane 10, Zn^{2+}.

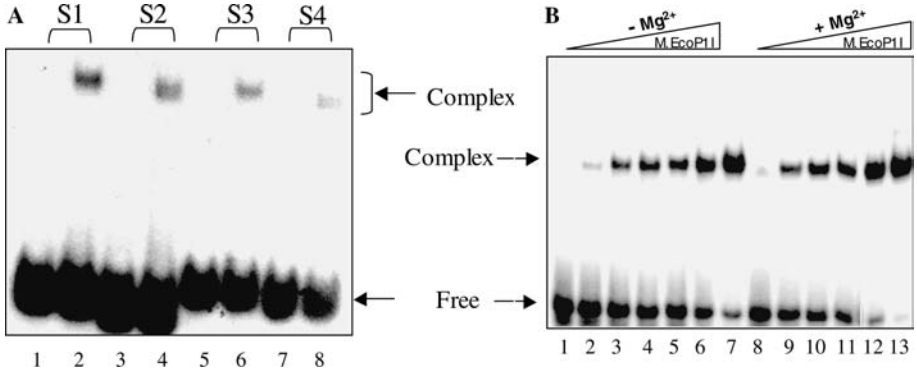

Fig. 2. Binding of EcoP1I DNA MTase to single-stranded DNA. (A) Different oligonucleotides (approximately 150 nM), S1, S2, S3, and S4 that differ from each other at the recognition sequences were incubated with EcoP1I DNA MTase (1 μM). While S1 contains the cognate sequence, AGACC, S2 is complementary with GGTCT in its sequence. S3 has an AGTCC instead of AGACC, in the same position as the latter and S4 has its complementary sequence, GGACT. The formed complexes were analyzed as described in Materials and methods. Lanes 1, 3, 5, and 7, no protein; lanes 2, 4, 6, and 8, 1.5 μM M.EcoP1I. (B) Effect of Mg^{2+} on the binding of M.EcoP1I to single-stranded DNA. The 5′-end-labeled oligonucleotide 1 (approximately 100 nM) and increasing amounts of EcoP1I DNA MTase were incubated in binding buffer (without or with Mg^{2+}) for 10 min and analyzed as described in Materials and methods. Lanes 1–7 (buffer without Mg^{2+}) and lanes 8–13 (buffer with Mg^{2+}). Lane 1, no protein; lanes 2 and 8, 0.136 μM; lanes 3 and 9, 0.272 μM; lanes 4 and 10, 0.34 μM; lanes 5 and 11, 0.54 μM; lanes 6 and 12, 1.36 μM; and lanes 7 and 13 2.72 μM.

the complementary or non-cognate sequences (Fig. 2A, compare lanes 2, 4, 6, and 8). DNA binding assay was also performed in the presence of AdoMet and the ternary complexes were analyzed on native PAGE. Sequence discrimination is not affected by the presence of AdoMet (data not shown). DNA binding assays were performed both in the presence and absence of Mg^{2+}. As can be seen from Fig. 2B, M.EcoP1I binds single-stranded DNA in the absence and presence of Mg^{2+}. Presence of Mg^{2+} enhanced the ssDNA binding ability of the enzyme (Fig. 2B). The apparent K_d value in the absence of Mg^{2+} was calculated to be 1.5 μM while in the presence of Mg^{2+} it was 0.75 μM. As mentioned earlier, gel shift assay is not an equilibrium method, therefore true K_d values cannot be determined by this procedure. Other metal ions did not affect the DNA binding ability of the enzyme (data not shown).

Binding affinities of EcoP1I methyltransferase to single- and double-stranded DNA were further investigated by performing a competition experiment. As can be seen from Figs. 3A and B, respectively, both single- and double-strand DNA–protein complexes were competed out by single-stranded DNA. The complex with double-stranded DNA was competed out to a greater extent than the complex with single-stranded DNA (compare lanes 2–5 of Figs. 3A and B), suggesting that the enzyme has greater affinity of binding to single-stranded DNA than double-stranded DNA. The nature of the slowly migrating complex in Fig. 3A, lane 1, is not known but it may be a specific complex as it disappeared upon addition of increasing concentration of ssDNA. Binding of single-stranded DNA by M.EcoP1I was unexpected but not unprecedented. As mentioned earlier, a few DNA MTases, which methylate ssDNA such as BcnI and DpnII, must also bind ssDNA [2,3].

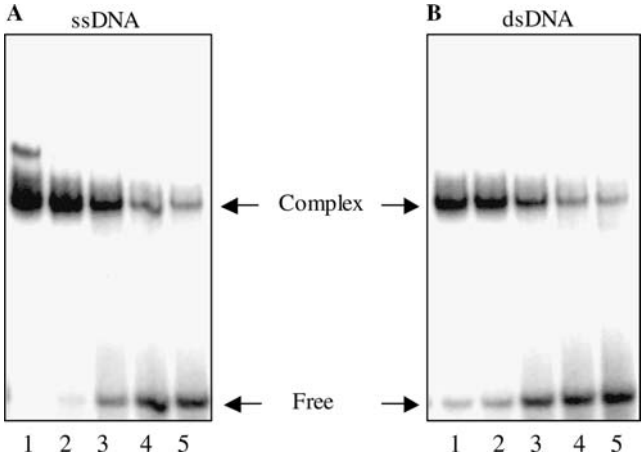

Fig. 3. Effect of competitor 72 mer ssDNA on binding of EcoP1I DNA MTase to single-stranded and double-stranded DNA. (A) 5′-end-labeled oligonucleotide 1 (approximately 100 nM) was incubated with EcoP1I DNA MTase (1.36 μM) on ice for 10 min in the presence of increasing concentration of unlabeled oligonucleotide 1 and analyzed as described in Materials and methods. Lane 1, no unlabeled oligonucleotide; lane 2, 8.5 nM; lane 3, 42.6 nM; lane 4, 170.2 nM; and lane 5, 426 nM. (B) 5′-end-labeled duplex A (approximately 100 nM) was incubated with EcoP1I DNA MTase (1.36 μM) on ice for 10 min in the presence of increasing concentration of unlabeled oligonucleotide 1 and analyzed as described in Materials and methods. Lane 1, no unlabeled oligonucleotide; lane 2, 8.5 nM; lane 3, 42.6 nM; lane 4, 170.2 nM; and lane 5, 426 nM.

Methylation of single-stranded and double-stranded DNA

Binding to ssDNA made it imperative to investigate the possibility of single-stranded DNA methylation. Methylation assays were carried out using equimolar concentrations of M13mp18 ssDNA and pUC19 DNA. It is relevant to mention that while the former has four, the latter has three sites for the enzyme. The specific

Table 1
Methylation activities of M.EcoP1I and R.EcoP1I on single- and double-stranded DNA substrates

Enzyme	Specific activity (pmol min^{-1} mg^{-1})	
	pUC19 (ds)	M13mp18 (ss)
M.EcoP1I	0.9	1.2
R.EcoP1I	13.6	0.26

The enzymes were purified as described in Materials and methods and assayed for MTase activity using pUC19 supercoiled and M13mp18 single-stranded DNA as substrates. Methylation was monitored by the transfer of radiolabeled methyl group from [methyl-^{3}H]AdoMet. Results were averaged of three experiments.

activity of M.EcoP1I, for both double as well as single-stranded DNA, is almost the same (Table 1). EcoP1I restriction enzyme methylates single-stranded DNA, albeit to a much lesser extent than double-stranded DNA (Table 1). It may be argued that under the reaction conditions M13mp18 ssDNA may form certain double-stranded secondary structures and the MTase could have acted upon these pseudo-substrates. This possibility can be ruled out, since even EcoP1I restriction enzyme would have methylated at the same regions. The type III restriction enzymes do not cleave single-stranded DNA [29]. EcoP15I DNA MTase does not methylate single-stranded DNA (unpublished results).

Kinetic parameters of methylation were determined using M13 single- and double-stranded DNA as substrate. The rate of methylation was linear with increasing concentration of ssDNA upto 1.14 μM and plateaued around 2.85 μM (Fig. 4A). However in case of dsDNA, saturation was not seen at the highest DNA concentration used (5.17 μM) (Fig. 4B). The K_m for single-stranded DNA is 0.78 μM while that for double-stranded DNA is 0.68 μM. The enzyme has a k_{cat} value of 0.032 min^{-1} for ssDNA and 0.036 min^{-1} for dsDNA, indicating almost similar turnover of both the substrates. Studies done in our laboratory indicated that M13mp18 ssDNA could be methylated (Table 1) but not restricted by the enzyme, R.EcoP1I [30]. Interestingly, M.EcoP1I is the first methyltransferase of the type III R–M family that is shown to methylate single-stranded DNA. Methylation of single-stranded DNA by M.EcoP1I can be invoked to elucidate the mechanism of action of the enzyme. It may be recalled that EcoP1I recognizes the sequence 5′-AGACC-3′ and methylates the central adenine [31]. This indicates that only one strand of DNA is modified. However, the enzyme does bind both single- and double-stranded substrates.

This unique property of EcoP1I methyltransferase can be extended to explain the survival of filamentous single-stranded DNA phages of *E. coli*, such as fd and M13. These replicates in cells as double-stranded, plasmid-like DNA molecules, but a single strand only is incorporated into phage particles [32]. These phages are restricted normally in cells harboring type I and type II restriction enzymes, but in vivo are extremely resistant to the action of type III restriction enzymes even though the double-stranded, replicative form is a good substrate for these enzymes in vitro [33]. The resistance of single-stranded DNA to the type III R–M system is still not well understood.

Binding and methylation by EcoP1I methyltransferase leads to the phage single strand genome being methylated at all the recognition sequences for EcoP1I. It should be noted here that these enzymes have an asymmetric recognition sequence and thus only the 5′-AGACC-3′ present on the single strand will be

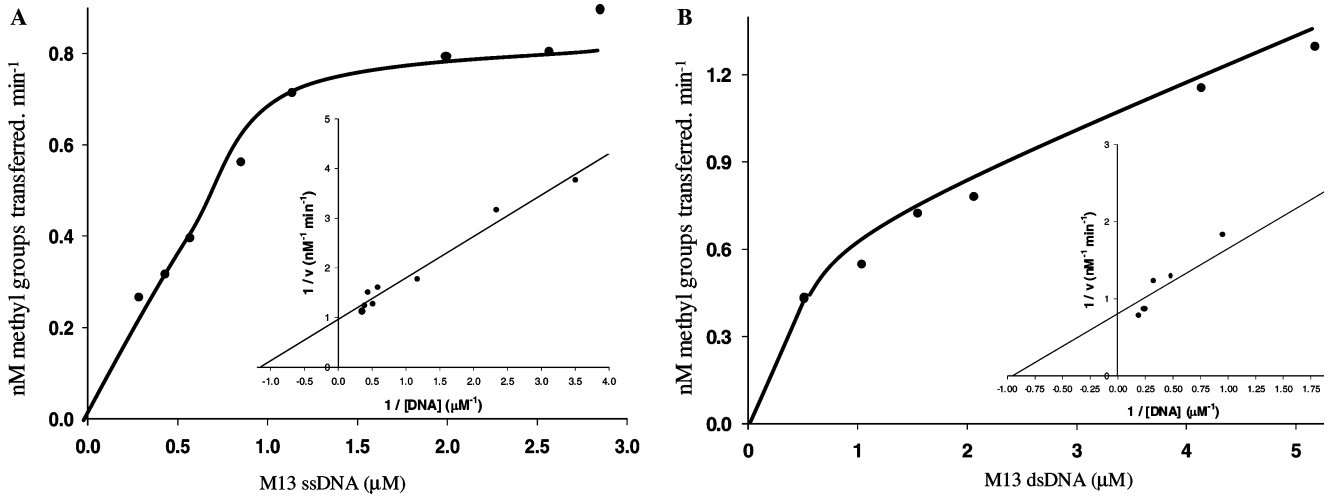

Fig. 4. Methylation of single-stranded and double-stranded DNA by M.EcoP1I. Methylation assays were performed using 0.034 μM M.*Eco*P1I and DNA concentration ranging from 0 to 2.85 μM (single-stranded) and 0 to 5.15 μM (double-stranded), as described in Materials and methods. Data were plotted using Sigma plot software. Lineweaver–Burk plots (insets) were used to calculate kinetic parameters. Results were averaged from three experiments for single-stranded DNA.

methylated and not the 5′-GGTCT-3′. As a fully modified recognition site for these enzymes has a methyl group on only one of the DNA strands [34], DNA replication results in one daughter molecule that has inherited the parental methyl group and is thus fully modified and a second daughter molecule that has no methyl group and is thus unmodified. If these sites remain unmodified for any length of time, they would be cleaved by the restriction enzyme. This would be lethal to the phage. One way of avoiding this would be, if modification of DNA is tightly coupled to replication. One could envisage the modification enzyme built into the replication complex. If this were the case, the single-stranded DNA of the phage could be modified during its conversion to the double-stranded form [29]. The mechanisms of single-strand DNA methylation and/or the enzyme being a part of the replication complex may coexist so as to ensure protection of the phage DNA from the type III restriction–modification systems in the cell. Methylation may occur upon injection of phage genome into the cell or during the course of replication as a part of the replication complex. Thus, methylation of single-stranded DNA by M.EcoP1I could be considered to be a part of an anti-restriction system, mechanisms which allow survival of phages in their bacterial hosts [34].

Acknowledgments

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