Processivity of Phage T4 Dam DNA methyltransferase (T4Dam)

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Abstract: Single turnover and pre-steady state (burst) kinetic analyses of the bacteriophage T4 Dam DNA-(N6-adenine)-methyltransferase (MTase)-mediated methyl group transfer from S-adenosyl-L-methionine (AdoMet) to Ade in DNA substrate containing one or two specific GATC sites with different combinations of methylated and unmodified targets were carried out. The single turnover experiment showed that the total amount of methylated DNA is approximately equal to the input DNA concentration (one unmethylated GATC site). We verified our results by performing the single turnover experiment on hemimethylated DNA and on DNA substrate that has two unmethylated GATC sites. The total amount of methylated product is approximately one-half the concentration of input DNA (hemimethylated)and is approximately double the concentration of input DNA in case of the substrate that has two unmethylated GATC sites. We supported our results further by using a burst magnitude approach. Based on our results, we can state that, T4Dam MTase does not do intrasite processivity and it could be able to methylate one adenine residue of unmethylated GATC site per single turnover.
[Hany Elsawy. **Processivity of Phage T4 Dam DNA methyltransferase (T4Dam).** *J Am Sci* 2013;9(7):340-346]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 42

Key word: T4Dam, enzyme processivity, enzyme kinetics.

1. Introduction:

DNA from various sources contains the methylated bases N6-methyladenine, 5-methylcytosine, and N4-methylcytosine in addition to the four standard nucleobases. These methylated bases are natural components of DNA which distinguishes them from a large variety of chemically modified bases that can be formed by alkylation or oxidative damage of the DNA.

DNA methylation is introduced enzymatically by DNA methyltransferases (MTases) after DNA replication. These enzymes use S-adenosyl-L-methionine (AdoMet) as the donor of an activated methyl group and modify the DNA in a sequence-specific manner, usually at palindromic sites, producing S-adenosyl-L-homocysteine (AdoHcy) and methylated DNA (Jeltsch 2002). The methylation does not interfere with the Watson/Crick pairing properties of adenine and cytosine but the methyl group is positioned in the major groove of the DNA, where it can easily be detected by proteins interacting with the DNA. Thereby, methylation adds extra information to the DNA that is not encoded in the sequence, and the methylated bases can be considered the 5^{th}, 6^{th}, and 7^{th} letters of the genetic alphabet.

Most prokaryotic DNA MTases are parts of restriction/modification (RM) systems, which are widely distributed in the bacterial and archeal kingdoms. These systems comprise two enzymes, a restriction endonuclease that specifically recognizes and cleaves DNA within short, often plaindromic sequences. The cellular DNA is protected from cleavage by a corresponding MTase, because it modifies the DNA within the same sequence and prevents endonuclease action. Thereby, the methylation pattern imprints a bar code on the DNA that allows the bacteria to distinguish between foreign and own DNA.

A second group of prokaryotic MTases is not accompanied by a restriction enzyme (solitary MTases, also called orphan DNA MTases) as exemplified by the E.coli DNA adenine methyltransferase (EcoDam) enzyme that recognizes the GATC palindromic sequence and methylates adenine at N6-position and the CcrM (cell cycle-regulated DNA MTase) enzyme, originally identified in *Caulobacter crescentus*, which methylates the adenosine in the sequence 5'-GANTC-3' (where "N" is any nucleotide) (Zweiger *et al.*, 1994). Instead, these enzymes are involved in diverse biological pathways such as gene regulation, mismatch repair, DNA replication and nucleoid structure determination (Casadesus & Low, 2006; Casadesus & Low, 2008).

One important property of DNA MTases is their processivity in the methylation of DNA molecules containing more than one target site. Processive enzymes stay bound to one DNA molecule after first turnover and methylate several target sites on that moleculewithout dissociation. Thereby, they directly convert unmethylated DNA into DNA modified at all target sites. Distributive enzymes, in contrast, always dissociate from the DNA after one methyl group transfer leading to an accumulation of methylation

intermediates, i.e. DNA molecules that are modified at some but not all target sites.

The processivity of DNA methyltransferases has a strong impact on their biological function, because DNA methylation is established in a radically different way by each type of enzyme. The EcoDam enzyme, for example, was shown to be highly processive, thus leading to efficient re-methylation of the GATC sites after DNA replication, although particular flanking sequences were shown to reduce processivity. Processivity is essential for methylation of the ~ 20000 GATC sites within the E.coli genome and the relatively few EcoDam molecules present in the cell (Szyf*et al.*,1984; Boye*et al.*,1992).

In general, all MTases that are accompanied by a restriction endonuclease show a distributive mechanism of DNA methylation,whereas all solitary MTases methylate DNA in a processive manner.

The distributive mechanism of DNA methylation is a crucial adaptation of DNA MTases in R-M systems to the biological function of these systems which is to cleave phage DNA by the restriction enzyme. It is important that the endonuclease reaches its site on the phage DNA before it is modified. It therefore makes sense that MTases are distributive which considerably lowers down the rate of DNA methylation, whereas endonucleases, in general, are processive in their mode of action.

T4Dam is Dam homologous without a restriction enzyme counterpart that present in phage T4. T4 and EcoDam may have a common evolutionary origin, sharing up to 64% sequence identity. T4Dam was shown to be processive (Zinoviev *et al*., 2003).

Coffin & Reich 2009 have demonstrated that EcoDam utilizes two forms of processive catalysis: intersite processivity whereby multiple GATC sites on the same DNA molecule are methylated and intersite processivity where the monomeric enzyme methylates both strands of a single unmethylated duplex prior to dissociating from the substrate. In this study we wanted to investigate if the T4Dam will do intrasiteprocessivity like its highly related enyzme (EcoDam).

2.The experimental Design:

I. Expression and purification of T4Dam

Bacterial Growth:

Escherichia coli GM 2971 (F^- mrr^- hsd S20 r_B m_{B-} ara14 proA2 lacY1 galK2 rspL20 (str^r) xyl5 mtl 1 supE44 dam13:Tn9 Cm^r) was from Dr. M. G. Marinus, Department of Pharmacology, University of Massachusetts Medical School, Worcester. Plasmid-containing cells were grown in LB-ampicillin broth at 30°C to an A^{600}= 0.8 - 1.0. The temperature was raised to 42°C, and incubation continued for 3 hrs. The cells were collected by low speed centrifugation and stored frozen at - 20°C.

Purification of T4Dam:

Frozen cells were suspended in 30 ml of PEM buffer (20 mM KPO_4, 1 mM EDTA, 7 mM 2-mercaptoethanol) with 0.1 mM phenylmethylsulfonyl fluoride, 0.1 % of Nonidet P-40, and 0.4 M NaCl. Lysozyme was added to a concentration of 500 µg/ml and, after a 1 h incubation at 4°C, the cells were disrupted by sonication. Insoluble debris was removed by centrifugation at 11,000 rpm for 30 min. The supernatant was passed through a DEAE-cellulose (DE52) column connected to a 1.5 × 10-cm P11 phosphocellulose column (equilibrated in buffer PEM with 0.2 M NaCl). Proteins bound to the P11 phosphocellulose were eluted in a 200 ml, 200 - 800 mM NaCl gradient in PEM buffer. DNA MTase activity was monitored as described below; appropriate fractions were pooled and dialyzed against PEM with 0.1 M NaCl and 50% glycerol and stored at -20°C. The concentration was calculated using a molar extinction coefficient at 280 nm of 14,700.

Preparation of DEAE-cellulose resin:

DEAE-cellulose (anion exchanger) was purchased from Sigma. 5g of DEAE-cellulose was slowly added to 300 ml of 0.1 M NaOH with gentle stirrer for 30 min (pH reached to 13). The NaOH solutionwas discarded and the resin washed with dd H_2O until pH reached to pH 8.0. Then solutionwas replacedwith 0.1 M HCl with gentle stirrer for 30 min (pH reached to 1.0). The resinwas washedwith dd H_2O until pH reached to 3.0. Water was discarded and replaced it with 10 x buffers (500 mMTris-HCl Ph 8.0) and gentle stirrer for 30 min. The 10x buffer was discarded and then equilibrated the resin with 50 mM Tris-HCl pH 8.0, degassed and the fines removed before the suspension of DEAE-cellulose resin was transferred into a glass column.

II. DNA MTase Assay:

Methyl transfer assays were carried out by a DE81 ion exchange filter assay (Rubin & Modrich 1977). Reactions contained 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM dithiothreitol, 400 µg/ml of BSA, 5 µM AdoMet (6 Ci/mmol mixture of unlabeled and [^{3}H] methyl-labeled, PerkinElmer Life Science), 50 nM T4Dam and 500 nM DNA. After incubation at 37°C, 10 µl reaction fractions were quenched with 10 µl of 1% SDS. 15 µl quenched fractions were spotted on DE81 filter paper. The paper was washed three times with a 50 mM KH2PO4 buffer, washed once in 80% ethanol, washed once in 95% ethanol, and dried in diethyl-ether; all washing steps were for 5 min. Papers were dried and submerged in Bio-Safe II scintillation fluid.

Tritium levels were quantified using a Beckman Coulter LS6500 scintillation counter. Counts wereconverted to methylated DNA product and plotted against time.

III. Substrate preparation

III.a. Substrate preparation for single turnover and steady state assays

All DNA oligonucleotides were ordered from Operon and resuspended in TE buffer (10 mM Tris Ph 7.5, 1 mM EDTA). All constructs were annealed in a 1:1 molar ratio by being heated at 95^{o}C for 10 min and then slowly cooled to RT. Proper product formation was verified by PAGE. Concentrations of all DNA constructs were determined by measuring the A_{260}.

IV. Steady state assay

Incorporation of tritiated methyl groups onto DNA was monitored by a filter binding assay as previously described (Reich & Mashhoon 1993). T4Dam was diluted in protein dilution buffer (20 mM KPO_4, 1 mM EDTA, 7 mM 2 mercaptoethanol, 0.1 M NaCl and 50% glycerol). Reactions were conducted at 37^{o}C, and mixtures contained 50 nM T4Dam in MRB, 0.2 mg/ml BSA, 25µM AdoMet (6 Ci/mmol mixture of unlabeled and [^{3}H] methyl-labeled, PerkinElmer Life Science), and 21 bp DNA (1000 nM) in a final volume of 100 µl. Mixtures were allowed to equilibrate at 37^{o}C prior to initiation of the reaction with DNA. Reactions were quenched with 10 µl of 1% SDS at a single time point, and 15 µl was spotted onto 2.5 cm Whatman DE81 circular filter papers. Filter papers were washed three times in 50 mM KH_2PO_4, once in 80% ethanol, once in 100% s ethanol, and once in diethyl ether for 5 min each. Papers were dried and submerged in BioSafeII scintillation fluid. Tritium levels were quantified in a Beckman-Coulter LS6500 scintillation counter. Counts were converted to methylated product and plotted versus time.

V. Single turnover assay

The incorporation of methyl groups under single-turnover conditions was monitored at 37^{o}C with DNA limiting and T4Dam and AdoMet in excess. Reactions took place in MRB with 0.2 mg/ml BSA, 400 nM DNA, 420 nM T4Dam, and 30 µM AdoMet in a total volume of 100 µ1. At 0, 20,30,45, 60, 120 240, and 300 s, 10 µL aliquots of the reaction mixture were removed and quenched in 10 µl of 1 % SDS. Fifteen microliters of the resulting mixture was spotted on DE81 filter paper. Samples were washed, dried, and counted as described above. Counts were converted to nanomolar methylated product and plotted versus time. The K_{chem} for each substrate (unmethylated, hemimethylated top and bottom) was found by fitting the data to a single exponential in Sigma Plot 6.1.

3. Results and Discussion

Expression and purification of T4Dam

T4Dam MTase expression and purification is illustrated in Fig. 1. SDS-PAGE analysis revealed a strongly enhanced band at the position expected for T4Dam (molecular mass= 30.4 kDa). A two-step chromatography procedure provided an excellent means to obtain highly purified T4Dam MTase.

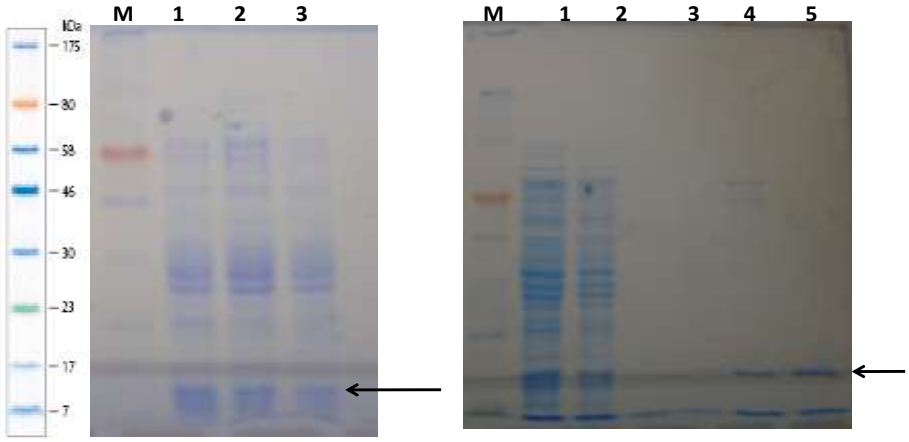


Fig. 1. Purification of T4Dam. Samples collected at each purification step were analyzed by 12% SDS-PAGE. For left gel, lane1, after sonification; lane2, supernatant after sonification and centrifugation at 11,000 rpm; lane 3, DEAE cellulose column flowthrough. For right gel, lanes 1-3, fractions from P11 column, lane 4, pool fraction of P11 phosphocellulose column; lane 5, after dialysis, lane M, protein standards (in kDa).

Intrasiteprocessivity.

T4Dam shows burst kinetics at 37°C with the catalytic step (k_{chem}=2.04±0.51min^{-1}) preceding a much slower rate-determining step (k_{cat}=0.16±0.05 min^{-1}), most likely product release. Single-turnover measurements with enzymes showing burst kinetics provide a direct measurement of the chemical step or any slower preceding steps. Our single turnover reactions were conducted at 37°C with limiting DNA (400 nM) and a very slight excess of enzyme (420 nM) with saturating AdoMet (30 µM). We observed a total amount of methylated DNA is approximately equal to the input DNA concentration (Fig. 2). That can be explained by T4Dam molecule may catalyze a methyl transfer and diffuse off the DNA before exchanging product AdoHcy for AdoMet. Our results are consistent with the results of T4Dam work by Zinoviev *et al.*, 2003. While, EcoDam, can catalyze two methyl transfer events occur per enzyme-substrate complex (intrasite processivity) (Coffin and Reich, 2009).

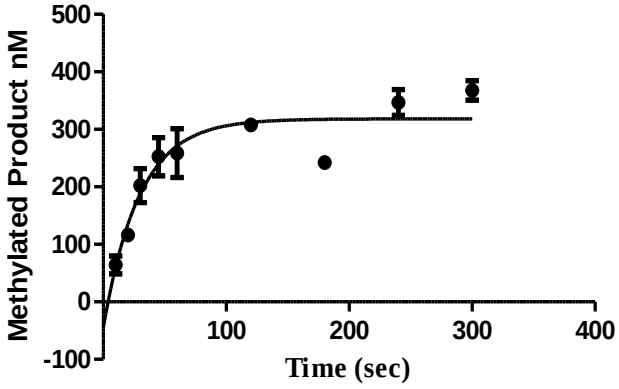


Fig. 2. Single-turnover analysis of unmethylated DNA reveals no intrasite processivity by T4Dam.

To verify our results: (i) we tested the same DNA substrate that was hemimethylated on the top strand (Fig. 3). We observed that the total amount of methylated product is approximately one-half the concentration of input DNA because of nonproductive binding events (Reich and Mashhoon, 1993).

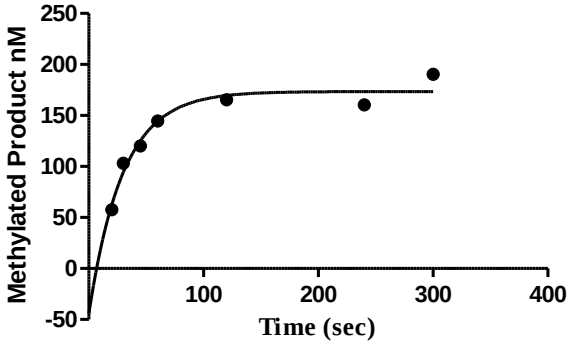


Fig. 3.Single-turnover analysis of hemimethylated DNA.

(ii) We performed the previous experiment with DNA substrate that has two unmethylated GATC sites (Fig. 4). We observed that the total amount of methylated product is approximately double theconcentration of input DNA andthat indicate the enzyme is methylating two adenine residues per single turnover.

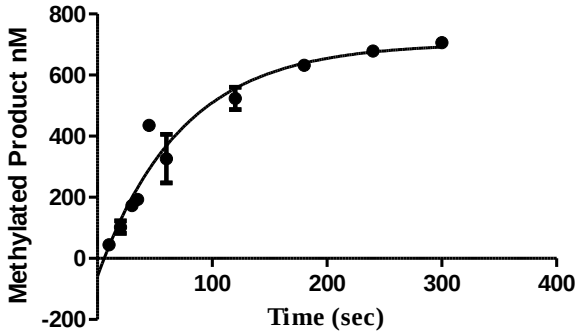
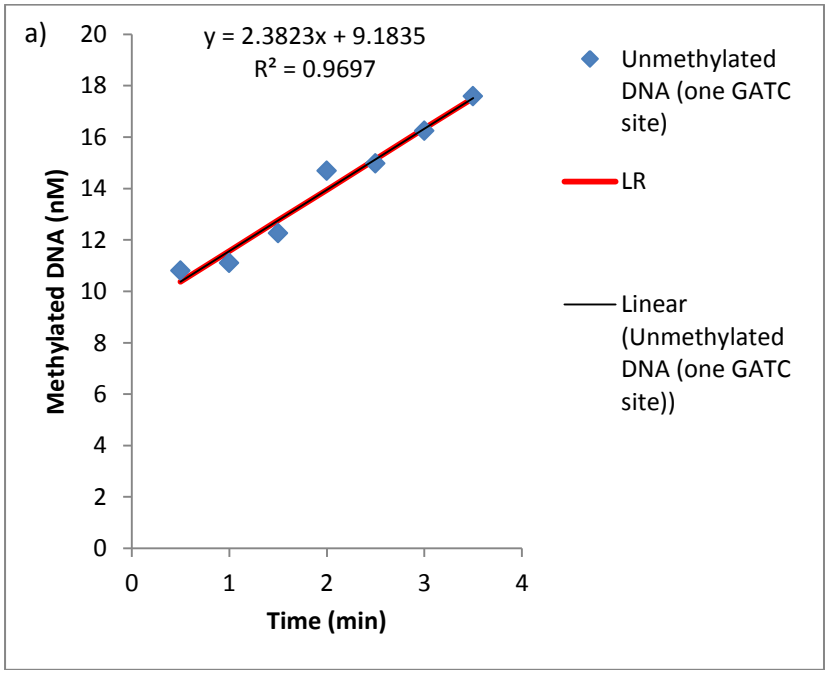


Fig. 4.Single-turnover analysis of DNA substrate containing two unmethylated GATC sites.

We supported our results further by using a burst magnitude approach. Burst reactions were conducted at 37°C with a very excess of DNA (200 nM), 10 nM enzymeand 5 µMAdoMet.

The methylation results with unmethylated DNA which contains only one GATC site showed burst value of about 10 (Fig. 5 a). This indicates that T4Dam is not able to reorient following methylation of one adenine residue (no intrasite processivity).

We measured burst values for DNA which is hemimethylated. As shown in Fig. 5 b, its burst value is about 10. The observed results here can best be explained by T4Dam being capable of rapid reorientation at hemimethylated sites as previously described (Zinoviev *et al.*, 2003). In addition, we measured burst values for DNA having two unmethylated GATC sites (Fig. 5 c) and showed burst value of about 20. This indicates that the enzyme is able to methylate two adenine residues with single turnover events.



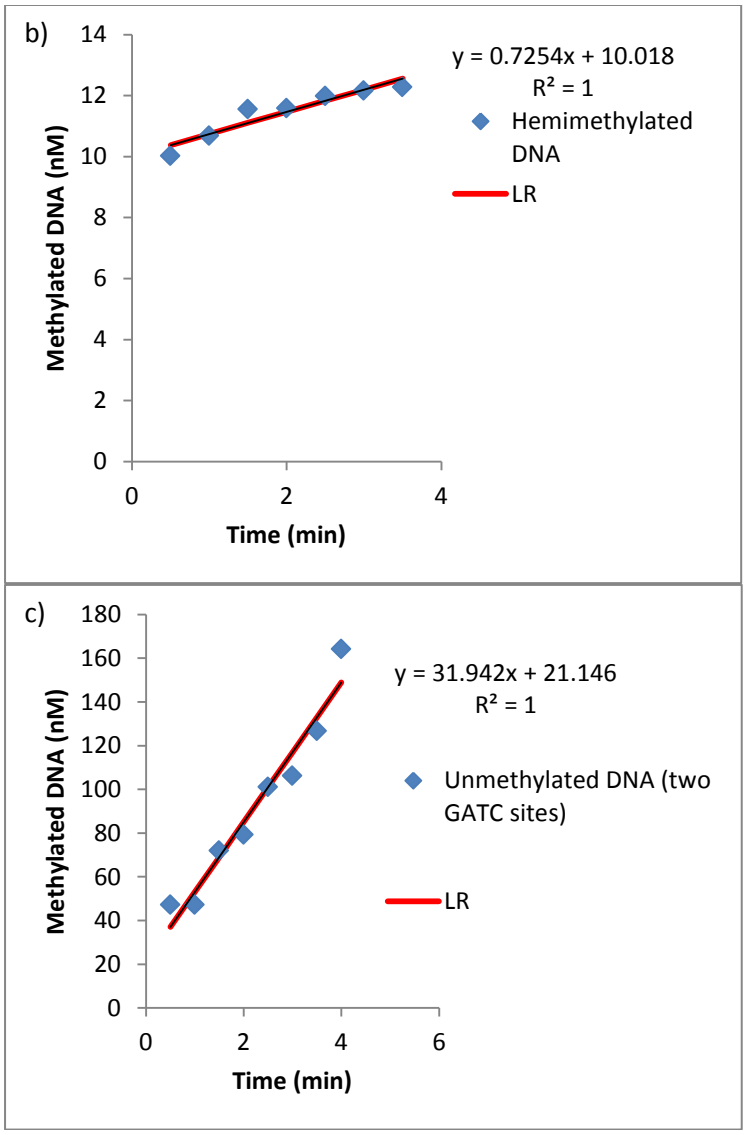


Fig. 5.kinetics of T4Dam methylation of (a)unmethylated (one GATC site), (b)hemimethylated and (c) unmethylated (two GATC sites) DNA.

Conclusions:

Based on the results presented, we draw the following conclusions. (i) T4Dam T4DamMTase does not do intrasite processivity. (ii) It can be able to methylate one adenine residue of unmethylated GATC site per single turnover.

Acknowledgements:

This work has been supported by a stipend of STDF-Egypt to H.E. I am grateful to Prof. Norbert Reich-University of California Santa Barbara (UCSB) for his help and hosting me in his lab. I also thank Reich's lab for their assistance.

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