Bacteriophage T4Dam DNA-(Adenine-\textsuperscript{N6})-methyltransferase

COMPARISON OF PRE-STeadY STATE AND SINGLE TURNOVER METHYLATION OF 40-MER DUPLEXES CONTAINING TWO (UN)MODIFIED TARGET SITES

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We analyzed pre-steady state and single turnover kinetics of bacteriophage T4Dam DNA-(adenine-\textsuperscript{N6})-methyltransferase-mediated methyl group transfer from S-adenosyl-l-methionine (AdoMet) to 40-mer duplexes containing native recognition sites (5\textsuperscript{\prime}-GATC/5\textsuperscript{\prime}-GATC) or some modified variant(s). The results extend a model from studies with single-site 20-mer duplexes. Under pre-steady state conditions, monomeric T4Dam methyltransferase-AdoMet complexes were capable of rapid methylation of adenine residues in 40-mer duplexes containing two sites. During processive movement of T4Dam to the next site, the rate-limiting step was the exchange of the product S-adenosyl-l-homocysteine (AdoHcy) for AdoMet without T4Dam dissociating from the duplex. Consequently, instead of a single exponential rate dependence, complex methylation curves were obtained with at least two pre-steady state steps. With 40-mer duplexes containing a single target site, the kinetics were simpler, fitting a single exponential followed by a linear steady state phase. Single turnover methylation of 40-mer duplexes also proceeded in two stages. First, two dimeric T4Dam-AdoMet molecules bound, and each catalyzed a two-step methylation. Instead of processive movement of T4Dam, a conformational adaptation occurred. We propose that following methyl transfer to one strand, dimeric (T4Dam-AdoMet)-(T4Dam-AdoHcy) was capable of rapidly reorienting itself and catalyzing methyl transfer to the target adenine on the complementary, unmethylated strand. This second stage methyl transfer occurred at a rate about 25-fold slower than in the first step; it was rate-limited by Dam-AdoHcy dissociation or its clearance from the methylated complementary strand. Under single turnover conditions, there was complete methylation of all target adenine residues with each of the two-site 40-mer duplexes.

DNA methylation is extremely important for the control of such processes as transcription, genomic imprinting, regulation of development, restoration of the correct structure of DNA, and chromatin organization (1). In contrast to most prokaryotic DNA methyltransferases (MTases),\textsuperscript{1} the Dam MTase is not a component of a restriction-modification system; rather, Dam methylation has other important cellular functions such as methyl-directed mismatch repair (reviewed in Ref. 2) and bacterial virulence (3). In the latter instance, despite the presence of a large number of virulence factors for intestinal bacteria (>20), it was sufficient to switch off the gene for the Dam DNA MTase to obtain avirulent live vaccine. Like all natural MTases, Dam MTases use the universal donor, S-adenosyl-l-methionine (AdoMet), transferring methyl groups to the Ade residues in the palindromic sequence 5\textsuperscript{\prime}-GATC-3\textsuperscript{\prime} (1).

Earlier, we carried out kinetic analyses of T4Dam-mediated methyl group transfer to oligodeoxynucleotide duplexes containing one or two specific GATC sites with different combinations of (un)methylated targets (reviewed in Ref. 4). The results for ligated 40-mer duplexes with those of the mixtures of the two unligated duplexes used to generate the 40-mer were compared (5). The salient results are summarized as follows: (i) T4Dam MTase modifies the 40-mer duplexes in a processive fashion. (ii) During processive movement on DNA from one site to the next, T4Dam was capable of rapidly exchanging product S-adenosyl-l-homocysteine (AdoHcy) in the ternary complex for substrate AdoMet. (iii) The processive steps of T4Dam action were consistent with an ordered bi-bi mechanism AdoMet \textdownarrow DNA \downarrow DNA\textsuperscript{M6} \downarrow AdoHcy \uparrow . However, in contrast to the steady state, here DNA\textsuperscript{M6} \uparrow signifies the departure of T4Dam from a methylated site GMTC \uparrow without physically dissociating from the DNA molecule (M denotes \textsuperscript{N6}-methyladenine, m\textsuperscript{6}Ade). (iv) Following methyl transfer at one site and linear diffusion to a hemimethylated site, T4Dam was capable of rapidly reorienting itself to the (productive) unmethylated strand. (v) The inhibition potential of fully methylated sites 5\textsuperscript{\prime}-GMTC/5\textsuperscript{\prime}-GMTC was much lower in a long DNA molecule as compared with short single-site duplexes. (vi) The T4Dam structural state depended on the molar ratio of the enzyme/duplex (6). If [T4Dam] < [DNA] ((pre)-steady state conditions), then the enzyme was monomeric. In contrast, if [T4Dam] > [DNA] (single turnover conditions), then the enzyme was mainly

\textsuperscript{1}The abbreviations used are: MTase, methyltransferase; AdoMet, S-adenosyl-l-methionine; AdoHcy, S-adenosyl-l-homocysteine; hmCyt, 5-hydroxymethylcytosine.

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dimeric; however, both enzymatic forms were catalytically active (7), but they had different kinetic characteristics. Since the results summarized above were based primarily on steady state and single turnover methylation studies (5), the present work was undertaken to determine whether methylation under pre-steady state conditions would yield results consistent with the kinetic model we proposed previously (8).

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—[3H-CH3]-AdoMet (15 Ci/mmol; 1 mCi/ml) was purchased from Amersham Biosciences. Unlabeled AdoMet (Sigma) was purified further by chromatography on a C18-reversed-phase column as described previously (9). Oligodeoxynucleotides (see Table I) were synthesized by Integrated DNA Technologies Inc. (Corvallis, IA), and their concentrations were determined spectrophotometrically. Duplexes were obtained by heating and annealing complementary oligodeoxynucleotide chains from 90 to 20 °C over 7–12 h. The 40-mer duplexes were constructed from shorter component duplexes using T4 polynucleotide kinase and T4 DNA ligase (SibEnzyme). All 40-mer duplexes had the same general structure except for the combinations of unmodified/modified Ade residues in the four 5′-GATC-3′ sites. For example, in the ligated 40-mer duplex shown below, the numbers in quotation marks (odd for upper strand and even for the bottom strand) mark the four potential methylation targets. Further information about the structures of the sites is provided in Tables I and II below.

“1” “3”

5′-CAGTGTAGGATCCTTCGGATCGATAAGGATCTCGGT-3′

3′-GCTAAACCTTAGGTAAGCCTATTCGTACGACCCA-5′

“2” “4”

SEQUENCE I

40-mer duplexes are presented in Fig. 1 and Table I. It is clear that among all the curves, only those for duplexes 40E and 40F had a classical form with a fast exponential phase accompanying by linear part of the curve, which can be described by an empirical equation.

\[
[3H-DNA]/[enzyme] = P(1 - e^{-k_{cat}t}) + k_{cat}t \quad \text{(Eq. 1)}
\]

Here \( P \) is the burst of product normalized to [enzyme], \( k_{meth1} \) is the rate of methyl transfer, and \( k_{cat} \) is the steady state reaction rate constant. The curves for 40-mer duplexes 40A–40D are more curvilinear and are best described by a more complex kinetic equation, which includes at least two exponential phases. We tried fitting the experimental data to an equation that describes a two-step burst reaction (13) that then continues into a steady state phase.

\[
[3H-DNA]/[enzyme] = P_1 - (P_1k_{meth1} - P_2k_{meth2})e^{-k_{meth1}t}/P_1k_{meth1} - (P_2k_{meth2} - k_{cat}) + k_{cat}t \quad \text{(Eq. 2)}
\]

Here \( k_{meth1} \) is the rate constant for methylation of the first target Ade residue, and \( k_{meth2} \) is the methylation rate constant for methylation of the second Ade residue. \( P_1 \) is the methylation level/bound duplex attained after the first step of the burst reaction (resulting from enzyme molecules initially bound to the productive strand). \( P_2 \) is the total methylation level/bound duplex after both steps of the burst reaction have taken place, and \( k_{cat} \) is the rate constant of the steady state phase. As seen in Table I, according to the two-step model, during the burst, the first Ade residue was methylated at a relatively rapid rate as compared with that for the second Ade residue. For example, with duplex 40A having all four unmodified target Ade residues, modification of the first Ade residue proceeded rapidly \( (k_{meth1} = 1.49 s^{-1}) \), whereas the rate constant for methylation of a second Ade was 10-fold lower \( (k_{meth2} = 0.15 s^{-1}) \). In the first step of the burst, there was methylation of one Ade residue/bound 40-mer duplex \( (P_1 = 1.05) \), and almost two Ade residues were methylated \( (P_2 = 1.83) \) upon completion of the burst. As postulated earlier (5), the second step of the burst reaction reflects a movement of T4Dam-AdoHcy from the methylated site to a nonmethylated sequence, exchange of AdoHcy for AdoMet, movement of the T4Dam-AdoMet complex to a next specific site, and then methyl transfer. The rate of this combined process \( (k_{meth2} = 0.15 s^{-1}) \) was 10-fold lower than that of the first burst phase but 10-fold higher than steady state rate \( (k_{cat} = 0.01 s^{-1}) \).

Methylation of duplex 40B, containing one unmodified (A/A) and one hemimethylated (M/A) site, proceeded somewhat differently. The rate constant of the first burst \( (k_{meth1}) \) was approximately the same as for duplex 40A, and the methylation level \( (P_2) \) was 0.66. However, the yield of the entire burst \( (P_2) \) was the same as for duplex 40A, although the rate constant of the second methylation phase \( (k_{meth2}) \) was 30-fold lower. Duplexes 40C and 40D contain two (un)methylated Ade residues located in cis or trans with respect to one another. These duplexes also showed a slight reduction in methylation level in the first reaction step \( (P_1 = 0.64 - 0.65) \), and the rate constant for the second methyl transfer was lower by about 25-fold. Surprisingly, methylation of duplex 40D (two target Ade residues on opposite chains in trans configuration) was about 2-fold faster in both burst steps as compared with duplex 40C, in which the two target Ade residues were in cis.

The reduction in rate constant for the second methylation step with 40-mer duplexes A–D may reflect the process of enzyme release/shift from the first site, exchange of AdoHcy for AdoMet, and methylation of the second Ade residue.

In contrast, the pre-steady state methylation kinetics of duplexes 40E (containing one fully methylated (M/M) site and one unmethylated (A/A) site) and 40F (containing one fully methylated (M/M) site and one hemimethylated (M/A)
site) were relatively simpler than for duplexes 40A–D. In fact, it was quite similar to methylation of a 20-mer duplex containing a single methylation site (13). Thus, methylation kinetics for duplexes 40E and 40F fit a curve corresponding to Equation 1, which describes the first step as a rapid, irreversible substrate conversion followed by a steady state phase. In both cases, the presence of an adjacent, completely methylated (M/M) site did not negatively affect methylation of the adjacent target Ade. Therefore, we can suppose that a T4Dam-AdoMet complex efficiently searches for the unmodified target Ade residue without binding the adjacent GMTC/GMTC sequence. Although the two duplexes gave burst values of about 0.9, the $k_{\text{meth}}$ value was 2-fold greater for duplex 40F. Further studies are necessary to determine whether T4Dam prefers hemimethylated sites.

Single Turnover Methylation of the 40-mer Duplexes—The experimental curves and kinetic parameters for single turnover methylation of the 40-mer duplexes are presented in Fig. 2 and Table I. As for 20-mer duplexes containing one specific site (7), the single turnover curves could not be fitted to a single expo-
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Table 1

| Duplex (A residues) | Pre-steady state | Single turnover |
|--------------------|-----------------|----------------|
|                    | \( k_{\text{meth1}} \) | \( k_{\text{meth2}} \) | \( k_{\text{cat}} \) | \( k_{\text{meth1}} \) | \( k_{\text{meth2}} \) | \( k_{\text{cat}} \) |
| 40A                | 1.05 ± 0.15     | 1.15 ± 0.13   | 0.01 ± 0.02  | 2.65 ± 0.11  | 3.14 ± 0.06  | 4.00 ± 0.02  |
| - - -              |                |                |             |               |               |               |
| 40B                | 0.66 ± 0.05     | 1.01 ± 0.04   | 0.01 ± 0.006| 1.19 ± 0.07  | 1.13 ± 0.08  | 3.27 ± 0.008 |
| - - -              |                |                |             |               |               |               |
| 40C                | 0.07 ± 0.0032  | 0.20 ± 0.0024 | 0.007 ± 0.0007| 0.09 ± 0.003 | 0.03 ± 0.004 | 0.05 ± 0.007 |
| - -               |                |                |             |               |               |               |
| 40D                | 0.64 ± 0.07     | 1.72 ± 0.0032 | 0.016 ± 0.0004| 0.87 ± 0.06  | 0.17 ± 0.05  | 0.018 ± 0.009 |
| - - -              |                |                |             |               |               |               |
| 40E                | 0.089 ± 0.004   | 0.001 ± 0.01  | 0.07 ± 0.0097| 0.25 ± 0.02  | 0.24 ± 0.06  | 0.005 ± 0.005 |
| - - -              |                |                |             |               |               |               |
| 40F                | 0.002 ± 0.15    | 0.019 ± 0.001 | 0.09 ± 0.013 | 1.01 ± 0.07  | 0.79 ± 0.20  | 0.038 ± 0.007 |
| - - -              |                |                |             |               |               |               |

*For convenience, the methylation status only of Ade residues in the two GATC target sites is listed: A = Ade; M = m6Ade.

The kinetic characteristics of duplex 40B methylation were comparable with that of duplex 40A, although the \( P_1 \) and \( P_2 \) values were lower proportionally according to the content of unmodified Ade residues. Duplexes 40C, 40D, and 40E each contain two target Ade residues, but they are distributed differently in the two specific sites. Nevertheless, the methylation levels of the two reaction steps had similar \( P_1 \) and \( P_2 \) values. One difference is that values for the rate constants \( k_{\text{meth1}} \) and \( k_{\text{meth2}} \) with duplex 40C were ~2-fold higher. Thus, the combination of two target Ade residues on one strand may facilitate more rapid (and processive) methylation than with the other target combinations. In this regard, the target site configuration on duplex 40C mimics hemimethylated polymeric DNA produced by semiconservative replication.

Duplex 40F contains a single unmodified target Ade residue in a hemimethylated (M/A) site that is adjacent to a fully methylated site. Unexpectedly, the rate constant \( k_{\text{meth1}} = 2.14 \) for the first methylation step was the highest of all the duplexes while exhibiting the lowest level of methylation (\( P_1 = 0.25 \)) of all the duplexes. However, complete methylation was attained (\( P_2 = 1.32 \)). The occurrence of two-step single turnover kinetics with duplexes containing one unmodified target Ade residue has been discussed previously (7).

It is also interesting to compare the single turnover methylation parameters of duplexes 40E and 40F (Table II) with their component 20-mer duplexes (7). There was good agreement between the \( k_{\text{meth2}} \) values for the two 40-mer duplexes and their component 20-mer duplexes. As suggested previously (7), these values characterize methylation of the second strand, which is rate-limited either by Dam-(AdoHcy/AdoMet) dissociation or by its clearance from the methylated complementary strand. In contrast, the \( k_{\text{meth1}} \) values are 4–5-fold higher for the 40-mer duplexes. This may indicate a more rapid adaptation of the two T4Dam dimers to the longer duplexes to form a productive complex.

Finally, in all cases, \( P_2 \) values were somewhat higher than the theoretical limits of methylation of the studied duplexes. It is possible that some methylation of Ade residues takes place in the sequence 5'-GATG-3' located in the middle of 40-mer duplexes. Recently, it was shown that such a sequence can be methylated by T4Dam, albeit at a reduced rate (16).

**DISCUSSION**

The main objective of the present work was to compare T4Dam MTase pre-steady state ([E] \( \ll \) [D]) versus single turnover ([E] > [D]) methylation in vitro of various 40-mer duplexes having two target sites. The distinction between the single turnover versus (pre)steady state reaction is that T4Dam functions as a dimer under single turnover conditions, whereas monomeric T4Dam functions under (pre)steady state conditions (7). Since our prior studies focused mainly on steady state and single turnover methylation, the present work was undertaken to determine whether the results under pre-steady state conditions would be consistent with our kinetic model (5).

**Pre-steady State Methylation Catalyzed by Monomeric T4Dam**—The curvilinearity of the plots for pre-steady state methylation of duplexes 40A–D dictates a kinetic model in which there is more than one exponential step. A fitting of the
experimental curves for these substrates by the Scientist and DinaFit programs showed that the model selection criterion values were comparable for reaction schemes with two or three exponential steps in the methylation burst. Calculations of the two-step and three-step model probabilities by means of DinaFit showed in all cases close to a 100% advantage of the two-exponential model. Thus, the principle of “Occam’s razor” (included in the program) chose the simplest model described by

**FIG. 2.** Single turnover kinetics of T4Dam methylation of 40-mer duplexes A–F. The concentrations in the resulting mixtures were as follows: 500 nM T4Dam and 50 nM 40-mer duplex (total concentration of specific sites is indicated); [3H-CH3]AdoMet was at 5 μM. The data were fit to Equation 3.
the experimental data. Table I shows that the total burst value ($P_2$) for duplexes 40A–D was almost 2. This agrees well with the results for methylation under steady state conditions, and it supports the kinetic model proposed earlier (5). This model proposed that following the first methyl transfer, T4Dam-produc

Table II

| Duplexa  | Status of Ade residues in site | $k_{\text{meth}1}$ | $k_{\text{meth}2}$ | $P_1$ | $P_2$ |
|---------|-----------------------------|-------------------|-------------------|------|------|
| 20-mer 1| -A                          | 0.21              | 0.023             | 1.03 | 1.90 |
|         | -A                          | 0.02              | 0.003             | 0.05 | 0.03 |
| 40E     | -M–A                        | 0.79              | 0.022             | 0.81 | 1.89 |
|         | -M–A                        | 0.09              | 0.007             | 0.04 | 0.21 |
| 20-mer 1m| -M                          | 0.37              | 0.039             | 0.45 | 1.01 |
|         | -A                          | 0.06              | 0.005             | 0.06 | 0.02 |
| 40F     | -M–M                        | 2.14              | 0.038             | 0.14 | 0.76 |
|         | -M–A                        | 0.64              | 0.005             | 0.02 | 0.04 |

a The two 20-mer duplexes are described in Ref. 7.

It was suggested that because of its asymmetric nature, hemimethylated DNA resulting from replication is the in vivo substrate for monomeric DNA MTases (21). However, this might simply result from an in vivo stoichiometry of [enzyme]/[sites] ≪ 1.0. In this regard, most DNA-(adenine-N6)-MTases appear to be functionally active monomers in vitro (22). However, Rsrl DNA MTase from Rhodobacter sphaeroides (23) and two MTases from Streptococcus pneumoniae (24) exist as dimers in solution. The Caulobacter crescentus DNA-(adenine-N6)-MTase (CcrM) is dimeric at physiological concentrations but is active as a monomer; a possible in vivo role for dimerization as a means to stabilize CcrM from premature catabolism was proposed (25). Recently, it was shown that KpnI DNA-(adenine-N6)-MTase from Klebsiella pneumoniae is a dimer in solution (26), as suggested by the nonlinear dependence of methylation activity on enzyme concentration.

Although MspI DNA-(cytosine-C5)-MTase is dimeric at high protein concentrations, this appears to reflect enzyme aggregation rather than functional activity (27). Human placental DNA-(cytosine-C5)-MTase forms a dimer in solution that is active in methylation in vivo. An additional insight into protein-protein interactions follows from the structural study of a mutant of HhaI DNA MTase, in which the dimeric state in crystal lattices and solution was shown (29). Moreover, comparison with other structurally characterized DNA MTases (HaeIII, human Dnmt2, PvuII, TaqI, Rsrl, MboII, and T4Dam) demonstrated that they also can be found as a 2-fold related dimer, although a general dimer configuration is individual and different in each case (29).

Thus, the old postulate that DNA MTases are active as monomers is not so strict, and dimeric MTases as potential natural catalysts have to be studied in more detail.

We conclude that T4Dam MTase activity and function...
in vitro are affected by its oligomeric state. In the monomeric form, the reaction occurs under pre-steady state conditions in which the T4Dam MTase-AdoMet complexes rapidly methylate target Ade residues in the 40-mer duplexes containing two sites, even when one of the sites is fully methylated. As the T4Dam MTase processively moves to adjacent sites without dissociating from the DNA duplex, the exchange of AdoHcy product for the AdoMet substrate in the ternary complex becomes rate-limiting. This explains the multiple exponential rate dependence due to the presence of at least two pre-steady state steps in the methylation reaction. In complexes containing a single target site, the linear steady state phase followed a single exponential step. In the dimeric form, in which the reaction takes place under single turnover conditions, two T4Dam molecules bind a 40-mer duplex and catalyze a two-step methylation at each site. There is clearly no processive movement of the enzyme but rather a conformational change, as described for the single turnover methylation of the 20-mer specific complexes (7). Complete methylation of all target Ade residues in the 40-mer target site duplexes occurs during single turnover reactions and following methyl transfer on one strand; the dimeric form of the enzyme is capable of rapidly reorienting itself to methylate the unmethylated strand.

T4Dam DNA Methylation in Vivo—It should be noted that the DNAs of phages T2, T4, and T6 are unusual in that they contain 5-hydroxymethylcytosine (hmCyt) in place of cytosine (30), and these hmCyt residues are modified further by glucosylation in a phage-specific pattern (31). Although T2 and T4 DNAs have m6Ade, T6 lacks any detectable DNA-adenine methylation (32) due to the fact that it appears to have no dam gene encoding the MTase (33). Taken together with the fact that null dam mutants of T2 and T4 lack detectable m6Ade (34), these results show that DNA-adenine methylation is not essential for phage viability and that the host Dam MTase is unable to methylate phage hmCyt-DNA. The latter is true whether or not the hmCyt residues are glucosylated (32). The natural in vivo substrate for phage Dam MTase methylation is the polymeric hemimethylated, hemiglicosylated region trailing the replication fork generated by semiconservative replication. However, glucosylation reduces the T2 and T4 m6Ade levels (32), suggesting that G-A-T-Glu-hmC (where Glu-hmC represents glucosylated hmCyt) is a poorer substrate than G-A-T-hmC; in addition, binding/movement of glucosyl transferase molecules on the DNA might compete with the action of the MTase. In this regard, glucosylation-defective T4gt mutants fully methylate all G-A-T-hmC sites prior to phage DNA maturation into virion particles (35). Thus, T4 Dam MTase is not limited during infection of a permissive host, although we do not know whether single turnover or steady state (or both) conditions prevail in vivo.

Since Dam methylation is not essential for phage viability, then what (if any) is its biological function? Contrary to the role of the Dam MTase of the host cell, there is no evidence that T4Dam functions in a methyl-directed mismatch repair system. This follows from the observation that the rate of spontaneous T4 mutation is unaffected in host mutH, mutL, mutS, and mutU (uvrD) mutants (36). Escherichia coli Dam methylation also plays a role in positive and negative regulation of cell gene transcription (37, 38). Thus, phage Dam methylation might modulate viral gene(s) expression. Although not essential for transcription, the ability to fine-tune the levels of mRNA could offer a slightly greater “fitness” to dam versus dam− phages. This might not be an issue in defined laboratory host strains, but in the wild, it might provide a selective growth advantage. To our knowledge, this question has not been addressed experimentally.

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