Dam methylase from *Escherichia coli*: kinetic studies using modified DNA oligomers: hemimethylated substrates

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ABSTRACT

We have measured steady-state kinetics of the N6-adenine methyltransferase Dam Mtase using as substrates non-selfcomplementary tetradecamer duplexes (d[GCCGGATCTAGACG]d[CGTCTAGATCCGGC]) containing the hemimethylated GATC target sequence in one or the other strand and modifications in the GATC target sequence of the complementary strands. Modifications included substitution of guanine by hypoxanthine (I), thymine by uracil (U) or 5-ethyl-uracil (E) and adenine by 2,6-diamino-purine (D). Thermodynamic parameters were obtained from the concentration dependence of the melting temperature (Tm) of the duplexes. Large differences in DNA methylation of duplexes containing single dI for dG substitution of the Dam recognition site were observed compared with the canonical substrate, if the substitution involved the top strand (on the GC rich side). Substitution in either strand by uracil (dU) or 5-ethyl-uracil (dE) resulted in small perturbation of the methylation patterns. When 2,6-diamino-purine (dD) replaced the adenine to be methylated, small, but significant methylation was observed. The kinetic parameters of the methylation reaction were compared with the thermodynamic free energies and significant correlation was observed.

INTRODUCTION

Type II restriction modification systems (R–M systems) consist of restriction endonucleases and their cognate type II DNA methyltransferases (Mtases). They represent very useful systems for studying the nature of DNA–protein interactions. Mtases recognise with high specificity short DNA sequences and catalyse the transfer of a methyl group from S-adenosyl-methionine (AdoMet), the methyl group donor, to the target DNA sequence (1). Three kinds of Mtases are known to exist in prokaryotes: C5-cytosine Mtases, N4-cytosine Mtases and N6-adenine Mtases (2–5).

The recent X-ray structure of the M-Hhal–AdoMet complex and that of the ternary M-Hhal–AdoMet–DNA complex (6) as well as the elucidation of a general mechanism of catalysis of C5-cytosine Mtases (7,8) constitute significant progress in the investigation of C5-cytosine Mtases.

In the case of N6-adenine Mtases, which catalyse the formation of N6-methyladenine, much less progress has been made. Biochemical studies on EcoRI Mtase (M-EcoRI) (9–13), M-Ecal (14,15), Dam Mtase (16–20) and M-Mval (21) have been reported. A mechanism for methylation by M-EcoRI has been proposed (12,22,23). Recently, the AdoMet-complex of an N6-A Mtase, M-TaqI, has been solved (24).

The interaction of EcoRI Mtase with hemimethylated DNA substrates containing base analogues has been investigated to characterise the contribution of individual functional groups toward DNA recognition by the enzyme (9–11). A similar approach has been applied in studies of M-EcoRI24I (13).

Contrary to other procaryotic Mtases, DNA adenine methyltransferase (Dam methylase) of *Escherichia coli* is not part of a R–M system (1–4). It recognizes specifically the sequence GATC in DNA and transfers a methyl group from AdoMet to the amino group of the adenine (25). This enzyme methylates DNA after replication, with a lag time (26), leaving the GATC sites of the DNA temporarily hemimethylated. The state of methylation (un-, hemi- or bimethylated) of the GATC site is a signal recognised by many systems in the cell: post-replicative mismatch repair, replication, transcription of certain genes, transposition and segregation of the chromosome (1,2,27,28). Although the biological role of Dam methylase is partially elucidated, little is known about its mechanism.

Dam methylase is a protein of 32 kDa molecular weight that acts as a monomer. It transfers one methyl group per binding event onto the GATC site (29). Methylation of the recognition sequence is modulated by the three base pairs flanking the two sides of the site (16). The interaction of the enzyme with its
recognition site must therefore be asymmetric. The mechanism of Dam methylase can be decomposed into three steps: specific recognition of the GATC site in the presence of the cosubstrate, AdoMet (17,18); methyl transfer from AdoMet to the adenine of the target site; release of the enzyme. These steps could, however, partially overlap. The specificity of Dam methylase could be determined by its binding to the site or the methyl transfer at this site. The cofactor also influences this specificity (17,18).

To approach the first aspect these points, we have undertaken steady-state kinetic studies of the enzyme with synthetic hemimethylated substrates, modified in the target sequence GATC in the complementary strand. The purpose of this study was to investigate whether (i) certain functional groups of the DNA target site are involved in the interaction with Dam Mtase and (ii) methylation of GATC shows an asymmetric pattern.

**MATERIALS AND METHODS**

**Design and properties of Dam Mtase substrates**

To be sure that no hairpin formation could occur, we have not used the tetradecamer previously utilised for the gel-retardation experiments (17). The following 14-base oligonucleotide duplexes were used as a reference:

- GM-C 5'-GGCGGMTCTAGACG-3'
- 3'-CGGCCTAGATCTGC-5'
- G-CM 5'-GGCGGTCCTAGACG-3'
- 3'-CGGCCCTMAGATCTGC-5'

All modified oligonucleotides were derived from these duplexes (see Table 1). Systematic substitutions in the central target sequence GATC were introduced: G→I, T→U, T→et 5

**Oligonucleotides**

The oligonucleotide substrates (Table 1) were synthesised by the phosphoramidite method. The β-cyanoethyl-phosphoramidites were purchased from Cytolab (Saint-Germain-en-Laye, France) or from Glen Research (Eurogentec, Seraing, Belgium).

**Synthesis of substituted β-cyanoethyl-phosphoramidite of deoxy-nucleoside derivatives.** β-cyanoethyl-phosphoramidite of deoxy-2-amino-adenine was synthesized according to the procedure described (30).

**Deoxy-5-ethyl-uridine.** Starting from 5-iodo-deoxy-uridine, 5-vinyl-deoxy-uridine was prepared following the method described by Heredewijn et al. (31). Catalytic hydrogenation yielded deoxy-5-ethyl-uridine as described (32). β-cyanoethyl-phosphoramidite of deoxy-5-ethyl-uridine was prepared using standard protocols (33,34). During the characterisation of oligonucleotides containing deoxy-5-ethyl-uridine it was found that the hydrolysis of these polymers with snake venom phosphodiesterase was very much lowered compared to that of the normal T-containing oligonucleotide. In a model study it was found that the dinucleotide dApdT was digested in 15 min, while dApdU<sub>et</sub> took 8 days for complete digestion (35). The resistance of these modified oligonucleotides towards snake venom phosphodiesterase is in agreement with reports in the literature (36–38).

**Methylation curves**

Methylation curves were measured in 0.1 M NaCl, 0.05 M phosphate buffer, pH 7.5. They were recorded at different concentrations between 30 and 80°C on a Perkin-Elmer spectrophotometer Lambda 15, equipped with a Peltier thermostatted cell holder and a temperature programmer, with a temperature raise of 1°C/min. Methylation temperatures (T<sub>m</sub>) were obtained from the peak of the derivative of the melting curves. Concentrations were determined after each melting curve from the 80° spectrum, using extinction coefficients obtained from the sum of those of the individual nucleotides. Concentrations varied from 0.03 to 0.001 mM in single strand.

**Enzyme preparation**

Enzyme preparation was as previously described (17). Enzyme concentrations were determined spectrophotometrically using the tyrosine and tryptophan absorbancies: ε<sub>520</sub> = 37 400 (39).

**Enzyme assays**

The methylation of DNA duplexes by Dam Mtase was studied in a buffer which is optimal for Dam Mtase activity (40): 50 mM HEPES/KOH pH 7.8, 1 mM DTT, 200 mM potassium glutamate, 5% glycerol, 0.2 mg/ml BSA.

Methylation of DNA duplexes by Dam Mtase was studied at various concentrations of DNA duplexes (see Table 1) to maintain steady-state conditions. Assays for methylation of oligonucleotides were performed as follows: 40 μl final volume in buffer contained 10 μM [methyl-3<sup>3</sup>CH<sub>3</sub>]-AdoMet (15 Ci/mmol, 67 μM, Amersham), varying concentrations of double-stranded oligonucleotide and 11.2 nM Dam Mtase (for G-CM, GM-CM, GM-CI, GM-CD, GM-CR, GM-CU, GE-CM, GM-CE duplexes) or 33.6 nM Dam Mtase (for GIU-CM, GM-CI, GIU-CM, GM-CD, GR-CM, GM-CR duplexes). Samples were incubated in a cryostat set at 6°C during t = 4 or 10 min (for GIU-CM, GM-CI, GD-CM, GM-CD, GR-CM, GM-CR duplexes). Two 10 μl aliquots of each concentration assay were withdrawn and spotted on DE81 (2.5 cm, Whatman) filters and dried for 10 min. Filters were placed vertically in a specially constructed filter rack with spacers which allowed good homogenous washing. The washing procedure (on a shaker) consisted (1000 ml for each washing) of one washing of 20 min and one of 10 min with 50 mM sodium phosphate buffer, followed by one washing in 95% ethanol (10 min) and finally ether. Filters were dried with a hairdryer until ether had evaporated. Filters were picked up with forceps, and then counted in scintillation vials (c.p.m.).

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For each substrate Michaelis–Menten, Eadie–Hofstee and Lineweaver–Burk plots have been obtained and the kinetic parameters were derived as an average from these data. A
minimum of three independent experiments were performed for each oligonucleotide duplex. For data treatment Kaleidagraph 3.0 software was used on a Macintosh IIvx computer.

For the determination of $K_m$ for AdoMet, 200 nM duplex G-CM and GM-C and 40 nM Dam Mtase were incubated with varying amounts of [methyl-$^3$H]-AdoMet.

**RESULTS**

**Melting studies on oligonucleotides**

Melting curves of the duplexes were measured as a function of concentration. The plots of the parent duplexes G-CM and GM-C, as well as that of the doubly methylated duplex GM-CM are shown in Figure 1. The equation $1/T_m = \Delta S/\Delta H - R \cdot \ln (c_t/4)/\Delta H$ was used (41-44) to determine the thermodynamic constants of the double strand dissociation reaction. The thermodynamic data are summarised in Table 1. Recalculation of the melting data in Table 1 to 100 nM duplex concentration yielded $T_m$ values between 9 and 30°C (last column, Table 1).

![Figure 1. Plot of ln[c(A)] versus 1/Tm for the canonical substrates G-CM (○), GM-C (■) and GM-CM (●).](image)

### Table 1. Thermodynamic parameters for the melting transitions of 14mer duplexes studied

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Duplex</th>
<th>$\Delta H^\circ$ [kcal-M$^{-1}$]</th>
<th>SD</th>
<th>$\Delta S^\circ$ [cal-M$^{-1}$K$^{-1}$]</th>
<th>SD</th>
<th>$\Delta G^\circ$ (25°C) [kcal-M$^{-1}$]</th>
<th>SD</th>
<th>$\Delta \Delta G^\circ$ [kcal-M$^{-1}$]</th>
<th>$T_m$ (°C) 100 nM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CM</td>
<td>5'-GCCGGMTCTAGACG-3' \ 3'-CGGCCTAGATCTGC-5'</td>
<td>111.5</td>
<td>5.6</td>
<td>338.4</td>
<td>16.9</td>
<td>10.6</td>
<td>0.5</td>
<td>-</td>
<td>25.7</td>
</tr>
<tr>
<td>G-CM</td>
<td>5'-GCCGGATCTAGACG-3' \ 3'-CGGCCTAGATCTGC-5'</td>
<td>66.5</td>
<td>4.9</td>
<td>200.9</td>
<td>14.8</td>
<td>6.6</td>
<td>0.5</td>
<td>0.0</td>
<td>9.2</td>
</tr>
<tr>
<td>GI-CM</td>
<td>5'-GCCGIATCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>101.6</td>
<td>6.6</td>
<td>308.0</td>
<td>20.0</td>
<td>9.8</td>
<td>0.6</td>
<td>3.2</td>
<td>23.5</td>
</tr>
<tr>
<td>GU-CM</td>
<td>5'-GCCGGUATCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>74.9</td>
<td>3.7</td>
<td>227.2</td>
<td>11.0</td>
<td>7.1</td>
<td>0.4</td>
<td>0.5</td>
<td>12.7</td>
</tr>
<tr>
<td>GIU-CM</td>
<td>5'-GCCGIUATCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>84.5</td>
<td>4.0</td>
<td>257.1</td>
<td>12.2</td>
<td>7.9</td>
<td>0.4</td>
<td>1.3</td>
<td>16.5</td>
</tr>
<tr>
<td>GE-CM</td>
<td>5'-GCCGGATCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>86.5</td>
<td>7.2</td>
<td>262.0</td>
<td>21.6</td>
<td>8.3</td>
<td>0.7</td>
<td>1.7</td>
<td>18.2</td>
</tr>
<tr>
<td>GD-CM</td>
<td>5'-GCCGGDTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>113.6</td>
<td>4.3</td>
<td>339.8</td>
<td>12.8</td>
<td>12.3</td>
<td>0.5</td>
<td>5.7</td>
<td>30.3</td>
</tr>
<tr>
<td>GM-C</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>81.4</td>
<td>4.6</td>
<td>245.6</td>
<td>13.8</td>
<td>8.2</td>
<td>0.5</td>
<td>0.0</td>
<td>17.3</td>
</tr>
<tr>
<td>GM-Cl</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>134.6</td>
<td>9.6</td>
<td>409.4</td>
<td>29.2</td>
<td>12.5</td>
<td>0.9</td>
<td>4.3</td>
<td>30.0</td>
</tr>
<tr>
<td>GM-CU</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>116.4</td>
<td>5.1</td>
<td>351.8</td>
<td>15.3</td>
<td>11.5</td>
<td>0.5</td>
<td>3.3</td>
<td>28.0</td>
</tr>
<tr>
<td>GM-CIU</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>104.6</td>
<td>4.8</td>
<td>318.6</td>
<td>14.6</td>
<td>9.5</td>
<td>0.4</td>
<td>1.3</td>
<td>23.0</td>
</tr>
<tr>
<td>GM-CE</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>83.9</td>
<td>5.3</td>
<td>253.8</td>
<td>16.1</td>
<td>8.2</td>
<td>0.5</td>
<td>0.0</td>
<td>17.7</td>
</tr>
<tr>
<td>GM-CD</td>
<td>5'-GCCGHTCTAGACG-3' \ 3'-CGGCCCTAGATCTGC-5'</td>
<td>99.0</td>
<td>4.0</td>
<td>295.4</td>
<td>10.9</td>
<td>1.9</td>
<td>0.5</td>
<td>2.7</td>
<td>27.6</td>
</tr>
</tbody>
</table>

$^a$Extrapolated from the equation $1/T_m = \Delta S/\Delta H - R \cdot \ln (10^{-7}/4)/\Delta H$. 

![image](image)
Kinetic studies

Taking into account the melting data of DNA duplexes and the well-known temperature sensitivity of the enzyme (16,17), it was decided to perform the methylation experiments with Dam Mtase at 6 ± 1°C. This avoided tedious corrections to take into account the fraction of duplex effectively present (9).

The initial rate of methylation of duplexes (duplex concentrations were 200 nM) measured in the presence of 3 µM AdoMet was linear with respect to enzyme concentration in the range from 0 to 40 nM M-Dam. Although the extent of methylation differed widely for the various duplexes linearity was observed in all cases (45).

Steady-state kinetic analyses of the Dam methylation of 200 nM DNA duplexes GM-C and G-CM (Fig. 2) was measured at different concentrations of [methyl-3H]-AdoMet and $k_{\text{cat}}$ values for AdoMet were determined to be 3.5 ± 0.2 µM and 1.5 ± 0.1 µM, for methylation of GM-C and G-CM, respectively. These values agree well with $K_m = 3.0 ± 1.5$ µM, determined previously for a different asymmetric 14mer (17).

Initial velocities of the M-Dam methylation of canonical and modified duplex substrates were determined at various concentrations of DNA duplexes and at a 3-fold concentration of $K_m$ of [methyl-3H]-AdoMet (10 µM). All reactions followed Michaelis-Menten kinetics (Fig. 3).

Dam Mtase was sufficiently active for DNA methylation at 6°C, as it follows from the kinetic parameters for the canonical substrates GM-C ($K_m = 45$ nM and $k_{\text{cat}} = 0.016 \text{ s}^{-1}$), G-CM ($K_m = 25$ nM and $k_{\text{cat}} = 0.021 \text{ s}^{-1}$) (Table 2) compared with analogous values determined for two other N6A Mtases: M-EcoRI [37°C, 14mer, $K_m = 2.44$ nM and $k_{\text{cat}} = 0.142 \text{ s}^{-1}$ (10,11)], M-EcAl (37°C, 24mer, $K_m = 170$ nM and $k_{\text{cat}} = 0.0034 \text{ s}^{-1}$ (14).

Replacement of dG by dl in the top strand increased the $K_m$ while the $k_{\text{cat}}$ value was decreased leading to a specificity constant $k_{\text{cat}}/K_m$ which was ~40 times lowered in GI-CM. If dl was introduced in the bottom strand, both $K_m$ and $k_{\text{cat}}$ were about the same as for the parent duplex GM-C, resulting in an only slightly lowered $k_{\text{cat}}/K_m$ for GM-Cl.

When thymine was replaced by uracil, the effects were quite different: dU for dT substitution in the top strand (GU-CM) halved the specificity constant, due essentially to the decrease of $k_{\text{cat}}$. In the bottom strand (GM-CU) the $K_m$ decreased only slightly, while $k_{\text{cat}}$ was doubled, resulting in the doubling of the specificity constant.

When both dl and dU were introduced in the top strand no reaction was measurable in (GIU-CM). Double substitution in the bottom strand (GM-CIU) gave a very small $k_{\text{cat}}$ for which a small $K_m$ was determined. This resulted in a specificity constant only one third of that of the parent duplex GM-C.

Substitution of 5-ethyl-uracil (dE) for thymine had rather small effect on all the kinetic parameters. The dE substituted duplexes appear to be good substrates, in particular the values of $K_m$ were virtually unchanged. Noteworthy is the lowering of $k_{\text{cat}}$ when the substitution is in the top strand.

The effect of the replacement of the methyl-accepting adenine residues by 2,6-diamino-purine (dD) came as a surprise: the effect is most notable in both strands with a much larger $K_m$ and much lower $k_{\text{cat}}$. In the top strand (GD-CM) $K_m$ increased ~6-fold, and $k_{\text{cat}}$ was about one fourth of that of the parent duplex, resulting in $k_{\text{cat}}/K_m$ lower by factor of ~25. In the bottom strand (GM-CD) the specificity constant $k_{\text{cat}}/K_m$ was lower by factor of ~10. The additional amino group in the minor groove does not appear to contribute to a stabilization of the base pair to the extent that it could not be methylated.

Upon suggestion of Dr M. Kohiyama (Institut Jacques Monod, Paris) that DNA–RNA hybrids during replication could contain hemimethylated GATC sequences, we have also measured hybrid complexes containing ribonucleotides in the unmethylated strand (for reason of stability we have used 2′-O-methylated ribonucleotides). In both cases, GR-CM and GM-CR, no reaction was observed.

DISCUSSION

We have attempted to characterise the contributions of the functional groups of the DNA substrate in the recognition by Dam Mtase. This was accomplished by using systematic substitutions in the non-selfcomplementary tetradecameric parent duplexes. The other goal of this study was to determine whether or not one of the other strand was significantly influenced by these substitutions and thus a preference of one over the other strand could be detected.
Table 2. Steady state kinetic parameters of Dam Mtae determined for the canonical 14mers and various substituted duplexes.

<table>
<thead>
<tr>
<th>Duplex</th>
<th>$k_m$ [nM]</th>
<th>SD</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>SD</th>
<th>$k_{cat}/k_m$ [s$^{-1}$.M$^{-1}$]</th>
<th>$[K_{m}]_{rel}$ [kcal.M$^{-1}$]</th>
<th>$[K_{m}]_{rel}$</th>
<th>$[k_{cat}/k_m]_{rel}$</th>
<th>RT.$\ln[k_{cat}/k_m]_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CM</td>
<td>25.5</td>
<td>5.2</td>
<td>0.0208</td>
<td>0.0045</td>
<td>816.34</td>
<td>1.000</td>
<td>0.00</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>GI-CM</td>
<td>51.9</td>
<td>7.5</td>
<td>0.0012</td>
<td>0.0003</td>
<td>23.12</td>
<td>2.035</td>
<td>+0.42</td>
<td>0.028</td>
<td>-2.13</td>
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<tr>
<td>GU-CM</td>
<td>15.2</td>
<td>4.9</td>
<td>0.0084</td>
<td>0.0021</td>
<td>550.44</td>
<td>0.596</td>
<td>-0.31</td>
<td>0.674</td>
<td>-0.24</td>
</tr>
<tr>
<td>GIU-CM</td>
<td>NR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GE-CM</td>
<td>23.3</td>
<td>15.4</td>
<td>0.0120</td>
<td>0.0056</td>
<td>516.45</td>
<td>0.914</td>
<td>-0.05</td>
<td>0.633</td>
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<tr>
<td>GD-CM</td>
<td>147.4</td>
<td>34.3</td>
<td>0.0044</td>
<td>0.0011</td>
<td>29.85</td>
<td>5.780</td>
<td>+1.05</td>
<td>0.037</td>
<td>-1.97</td>
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<tr>
<td>GR-CM</td>
<td>NR</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>GM-C</td>
<td>44.8</td>
<td>18.6</td>
<td>0.0155</td>
<td>0.0086</td>
<td>345.61</td>
<td>1.000</td>
<td>0.00</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>GM-Cl</td>
<td>47.4</td>
<td>6.9</td>
<td>0.0121</td>
<td>0.0031</td>
<td>254.92</td>
<td>1.058</td>
<td>+0.03</td>
<td>0.738</td>
<td>-0.18</td>
</tr>
<tr>
<td>GM-CU</td>
<td>33.2</td>
<td>3.9</td>
<td>0.0277</td>
<td>0.0072</td>
<td>835.34</td>
<td>0.741</td>
<td>-0.18</td>
<td>2.417</td>
<td>+0.53</td>
</tr>
<tr>
<td>GM-CIU</td>
<td>9.2</td>
<td>0.6</td>
<td>0.0010</td>
<td>0.0005</td>
<td>108.99</td>
<td>0.205</td>
<td>-0.94</td>
<td>0.315</td>
<td>-0.69</td>
</tr>
<tr>
<td>GM-CE</td>
<td>21.5</td>
<td>3.8</td>
<td>0.0140</td>
<td>0.0053</td>
<td>651.16</td>
<td>0.480</td>
<td>-0.43</td>
<td>1.884</td>
<td>+0.38</td>
</tr>
<tr>
<td>GM-CD</td>
<td>122.4</td>
<td>30.8</td>
<td>0.0041</td>
<td>0.0011</td>
<td>33.77</td>
<td>2.732</td>
<td>+0.60</td>
<td>0.098</td>
<td>-1.39</td>
</tr>
<tr>
<td>GM-CR</td>
<td>NR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Standard deviations were determined from a minimum of three independent determinations.

NR, no reaction

Thermodynamics of hemimethylated 14mer duplexes

The sequences studied are voluntarily highly asymmetric, because of the influence of neighbouring sequences on the directionality of Dam Mtae (16): on the left side the GATC sequence is flanked by four G-C pairs, while on the right side two weaker A-T pairs are followed by a G-C pair. In the case when methylation is on the A-T rich side the $\Delta H$ and $\Delta S$ values of duplex dissociation are lower then when methylation is flanked by G-C rich sequences. The destabilising effect of N6-methylation has been demonstrated to be due to the reduced opening and closing rates of the m6A-T base pair (46-48). Table 1 gives the values of the thermodynamic parameters obtained from semi-logarithmic plots of $\ln(c/4)$ versus $1/T_m$ (van't Hoff analysis). It is noteworthy that G-CM and GM-C have considerably different $\Delta H$ and $\Delta S$ values, although the central hemi-methylated target site is the same and has only been inverted. Simple nearest neighbour effects (42,43) are not sufficient to explain this difference. It is apparently energetically more difficult to melt the sequence from the left side than from the right half which contains the methylated adenine residue besides three A-T pairs out of seven.

Substitutions in the sequences studied gave irregular responses. In all cases increases in thermodynamical values were found (Table 1). This was the case, even for dI substituting for dG, contrary to the observations of Martin et al. (49) who had found that I-C base pairs were less stable than G-C and even than A-T pairs. In a recent study on hemimethylated oligonucleotides (11,50), however, the presence of I-C pairs instead of G-C pairs showed moderate to large increases in $\Delta H$ and $\Delta S$, depending on the sequence and the
The temperature sensitivity of our enzyme and consideration on conditions from ours (37°C versus 6°C). This difference between about one order of magnitude lower than that of M.EcoRI (10,11). The turnover number, $k_{cat}$, observed in the same range as that observed for other adenine Mtases, $K_m$, in the range between 250 and 315 K. They concluded that the compensation phenomena must be due to the properties of bulk water and differences in hydration states of the native and denatured form of a protein. For oligonucleotides these compensation temperatures $T_c$ are higher: for the tetradecamers studied here $T_c = 330.6$ K (57°C) for the melting and cooling data. This value is even higher ($T_c = 360$ K) for a series of nonamers (52), but of the same order ($T_c = 320$ K) for another series of tetradecamers (11).

Kinetics of methylation of modified hemimethylated 14mer duplexes

Dam Mtase followed Michaelis–Menten kinetics with the 14mer oligonucleotides G-CM and GM-C, as well as with the analogue-substituted duplexes. In some cases substrate inhibition was observed at high concentrations (over five times $K_m$) of substituted oligonucleotide duplex. The Michaelis constant, $K_m$, was in the same range as that observed for other adenine Mtases, like M.EcoRI (10,11). The turnover number, $k_{cat}$, however, was about one order of magnitude lower than that of M.EcoRI (10,11). It should be noted that these authors used different temperature conditions from ours (37°C versus 6°C). This difference between temperature shall account for the smaller $k_{cat}$ values observed. The temperature sensitivity of our enzyme and consideration on the $T_m$ of the 14mer duplexes, however, conditioned our choice of a lower temperature.

Previous work from this laboratory on Dam Mtase using high-resolution $^3$H-NMR (18) had demonstrated the stoichiometric binding of AdoMet to Dam Mtase, even in the presence of a 5-fold excess of the competitive inhibitor sinefungin. This was visualised by a small, quantitative change in chemical shift of the methyl-$^3$H resonance. Upon addition of the GATC-bearing oligonucleotide duplex, a rapid upfield change in the chemical shift, followed by a slow relaxation step to form the active ternary complex was found. This chemical shift was different from free or Dam-bound AdoMet. From these data and previous gel retardation experiments (17) it had been suggested that allosteric rearrangement took place upon addition of the DNA substrate to the preformed AdoMet-Dam Mtase complex. A similar conclusion was reached by Szilák et al. (15) for M.EcoRI. Very recently, Powell and Murray (54) demonstrated that AdoMet modified contacts with the DNA target sequence in a Type I Mtase, M.EcoK1.

Influence of analogue substitutions

Substitution of guanine by hypoxanthine. The difference between these two base residues is the absence of the 2-amino-group of guanine in the minor groove. A priori, it is expected that Dam Mtase acts primarily in the major groove where the methylation event takes place. This substitution is thus expected to have little influence on the activity of the enzyme, except by a possible destabilization of the duplex (42–44,55), but with little or no perturbation of the geometry of the duplex. This possibility should be minimal in view of the data in Table 1 and the low temperature at which the experiments have been executed. Thus the large effect of substitution of dl for dG in the top strand cannot be due to thermodynamic or structural causes. Reich and Danzitz (10,11) had concluded that significant interaction occurred between M.EcoRI and the DNA substrate in the minor groove.

Substitution of thymine by uracil or 5-ethyl-uracil. Here again the influence of these modifications on the stability of the duplexes should be minor (42–44), with little or no perturbation of the geometry of the duplex (56). Here, however, the fact that the thymine is paired with the methylated adenine and the proximity of the methyl groups in the double helix, small but significant effects may be expected. The importance of hydrophobic contacts in nucleic acid–protein interactions has been amply demonstrated. It was therefore of interest to probe the removal or increase of the hydrophobic methyl group on Dam Mtase activity.

Removal of the methyl group had a rather small effect, even improving the $k_{cat}$ of duplex GM-CU). This indicates that an interaction of the thymine methyl group with Dam Mtase is not important. The inverse operation, i.e. increasing the size of the 5-methyl-group by substituting it by an ethyl group gave a surprising result. In all cases this was a good substrate and was as well methylated as the parent duplexes. This means that, if there is the hydrophobic pouch which normally houses the thymine methyl group, it is sufficiently flexible to accept also a larger substituent.

Substitution of adenine by 2-amino-adenine (2,6-diamino-purine). This substitution adds an amino group in the minor groove. A priori, it is expected that Dam Mtase acts primarily in the major groove where the methylation event takes place. This substitution is thus expected to have little influence on the activity of the enzyme, except by a possible destabilization of the duplex (42–44,55), but with little or no perturbation of the geometry of the duplex. This possibility should be minimal in view of the data in Table 1 and the low temperature at which the experiments have been executed. Thus the large effect of substitution of dl for dG in the top strand cannot be due to thermodynamic or structural causes. Reich and Danzitz (10,11) had concluded that significant interaction occurred between M.EcoRI and the DNA substrate in the minor groove.
the case of M-EcoRI, although the effects were much more pronounced than observed for Dam Mtase.

**Importance of the strand location of analogue substitutions**

Although the differences are not very large, the effect of substitution is clearly more pronounced, if it takes place in the top strand. It should be remembered that the 14mer duplex utilised is asymmetric; it contains only G-C base pairs on one (left) side of the GATC target site and two A-T base pairs followed by a G-C pair on the other (right) side.

If one considers the ratio of \( K_m \) between the parent compound and the substituted duplexes as an estimate of the difference in standard free energy upon dissociation of the modified (m) and parent duplex (p) from their respective enzyme–DNA–substrate complex, one obtains

\[
\Delta G^* = \Delta G^*(p) - \Delta G^*(m) = RT \ln[K_m(m)K_p(p)] = RT \ln[K_m]_{rel}.
\]

The numerical values are found in the third last column of Table 2. If this free energy change is related to the thermodynamic stability of the duplexes studied, a plot of the thermodynamic \( \Delta G^* \) versus this free energy change \( \Delta G^* \) for each family of modified duplexes should show a straight line. This relationship appears to be reasonably followed by the duplexes modified in the top strand (Fig. 5, circles), but much less so in the case of the duplexes modified in the bottom strand (Fig. 5, squares).

Inversely, the relationship—even if it is not perfectly followed—indicates that lower thermodynamic stability favours the binding of the enzyme in the ternary complex. This is in agreement with the recent observations by Yang et al. (57) and Klimasauskas and Roberts (58) for M-Hhal binding to mismatched substrates.

Alternatively, the method introduced by Fersht (59) by comparing the \( k_{cat}/K_m \) values, i.e. the specificity constants, can be adapted to the present problem. Analogous to the above equation, one can write

\[
\Delta G_1 = \Delta G_1(p) - \Delta G_1(m) = RT \ln[(k_{cat}/K_m(m)) / (k_{cat}/K_m(p))] = RT \ln[k_{cat}/K_m]_{rel}.
\]

This relation reflects the interaction energy in the transition state of the active complex. The numerical values are found in the last column of Table 2. If the thermodynamic free energy change \( \Delta G^* \) (Table 1) is plotted versus transition state free energy \( \Delta G_1 \) again a reasonable correlation is observed, this time, however, with a positive slope, where only GM-Cl and GM-CU are ‘off the line. This suggests that the specificity of the interaction is related to the thermodynamic stability of the duplexes.

In this context the X-ray structures of M-Hhal, a C5-cytosine Mtase, may be of relevance (5,6). In the ternary complex oligonucleotide–AdoMet–M-Hhal, the cytosine to be methylated is swung out of the double helix and the ‘orphan’ guanine is maintained stacked by three hydrogen bonds with Gln 237: the main chain amide group of Gln 237 connects to O6 of the guanine base, while the imino- and one amino proton of guanine bind to the side chain amide-keto group of the same amino acid. From the published data (6), the latter hydrogen bond from the N2-amino group is 0.35 (12.5%) longer than the two others and thus considerably weaker. Little is indicated on the interaction of neighbouring bases with M-Hhal. Although M-Dam is not directly comparable with M-Hhal, since it is not a C5-cytosine Mtase, it is probable and had been suggested that a similar swing-out mechanism shall be operative with N6A Mtases (24) and an analogous stabilisation of the lonely complementary base. Such a mechanism could easily be envisaged: the T-A base pair is swung out of the double helix and the ‘orphan’ guanine is stabilized in an analogous manner as the guanine in M-Hhal (6).

Thus, the ‘orphaned’ thymine base in an N6A Mtase complex could be stabilized in an analogous manner as the guanine in M-Hhal. This, however, implies that changes in the base pair stability should inversely influence the stability of the active methylation complex; an increase in the stability of the base pair to be methylated should decrease its ability to open and swing out and thus form a complex with higher \( K_D \) and consequently \( K_m \) as had been observed (57,58).

Summarising the results of the interaction of Dam Mtase, with a variety of substrate analogues, we have observed that this enzyme is rather tolerant to different substitutions. It seems that in the case of Dam Mtase, may be in the case of other Mtases (14,21), DNA–protein interactions are not very tight, the \( K_m \) values being well in the 10–100 nM range. There are no reasons to propose pronounced conformational differences between the duplexes as was suggested for M-EcoRI by Reich and Danzitz.
(10,11) upon introduction of dl into their tetradecamer. Comparison of substrate properties of substitution in the top or bottom strand of the oligomers suggests that Mtase acts asymmetrically and very probably preferentially on the bottom strand. It also needs its recognition site to be surrounded by favourable flanking sequences, which is a common feature for interactions between DNA and Mtases (16,17,60) and restriction endonucleases (60).

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This paper is dedicated to Professor Emil Palecek (Brno) on the occasion of his 65th anniversary.

REFERENCES