Effect of 1-methyladenine on thermodynamic stabilities of double-helical DNA structures

Hao Yang, Sik Lok Lam *
Department of Chemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

Abstract
1-Methyladenine (m1A) alters T-A Watson–Crick to T-m1A Hoogsteen base pair. Owing to its conversion to N6-methyladenine (m6A) at higher temperatures, thermodynamic studies of m1A-containing DNAs using conventional melting methods are subject to the influence of m6A species. In this study, we applied nuclear magnetic resonance spectroscopy to determine the base pairing modes and effect of m1A on thermodynamic stability of double-helical DNA. The observed base pairing modes account for the destabilizing trend which follows the order T-m1A/C < A-m1A/C < C-m1A, providing insights into the m1A flipping process and enhancing our understandings of the mutagenicity of m1A.

1. Introduction
Methylation on DNA bases is vital for normal functioning of many biological processes. However, the presence of abundant environmental and endogenous alkylating agents leads to lethal and mutagenic damages which can halt replication and interrupt transcription [1,2]. Among possible alkylating sites of nucleobases, methylation at N1 of adenine leads to the formation of 1-methyladenine (m1A) which can block DNA replication [3]. Rather than involving DNA-glycosylases and DNA-methyltransferases [4], a DNA damage repair enzyme, AlkB, has been found to mediate a direct demethylation of m1A [5,6], providing a new repair pathway for DNA methylation.

Recently, crystallographic structural studies have revealed that the AlkB repair mechanism of m1A in both single-stranded DNA (ssDNA) [7] and double-stranded DNA (dsDNA) [8]. AlkB uses an unprecedented base flipping mechanism to access m1A in dsDNA and thus the energetic penalty to flip m1A is much higher than that in ssDNA [8]. Such findings provide a possible explanation for the observed repair preference of AlkB in ssDNA than dsDNA [6,9]. Through investigating the effect of m1A on dsDNA structures, we have shown that 1-methylation of adenine causes a switch of T(anti)-A(anti) Watson–Crick base pair to T(anti)-m1A(syn) Hoogsteen base pair [10] (Fig. 1). This formation of Hoogsteen base pair may affect the base flipping efficiency, providing structural insights into the m1A flipping process in dsDNA and enhancing our understanding of the AlkB repair process. Relaxation studies have also revealed that the inherent sequence-dependent conformational flexibility in DNA facilitates base extrusion during DNA methylation, thereby making base flipping energetically feasible [11]. In order to better understand the m1A flipping process, thermodynamic studies of m1A in dsDNA are needed. However, due to the feasible conversion of m1A to N6-methyladenine (m6A) at higher temperatures via Dimroth rearrangement [3,12], thermodynamic results from conventional melting methods such as ultra-violet (UV) or differential scanning calorimetry (DSC) are subject to the influence of m6A species, and thus the effect of m1A on the thermodynamic stability of dsDNA remains elusive.

Besides, mutagenicity studies have revealed that m1A prefers to pair with T over G, A, and C [3]. Yet the underlying reasons leading to such observed mutagenicity remain unclear. To further understand the mutagenicity and flipping process of m1A in dsDNA, the present work aims to (i) determine the effect of m1A on the
thermodynamic stability of dsDNA and (ii) rationalize the thermodynamic results through investigating and comparing the base pairing modes of G-m1A, A-m1A, and C-m1A with T-m1A [10] using high resolution nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Sample design

Fig. 1 shows our initial design of a 17-nt DNA sample, namely “Tm1A-oligo”, which contains a T-m1A base pair in the middle of the double-helical stem region [10]. The reference sample, “TA-oligo”, contains a T-A instead of a T-m1A base pair. The 5’-GAA loop was added to connect the two strands of the double-helix in order to simplify the sample preparation work. In this study, the T-m1A base pair was replaced by a G-m1A, A-m1A, and C-m1A base pair, and these DNA samples were named as “Gm1A-oligo”, “Am1A-oligo”, and “Cm1A-oligo”, respectively.

2.2. Sample preparation

All DNA samples were synthesized using an Applied Biosystems model 392 DNA synthesizer and purified using denaturing polyacrylamide gel electrophoresis (PAGE) and diethylaminoethyl Sephadex anion exchange column chromatography. For incorporating an m1A into the oligomers, 1-methyl deoxyadenosine phosphoramidite (ChemGenes Inc.) was used and the base deprotection step was performed at 37°C for 16 h. The necessary use of concentrated ammonium hydroxide in the deprotection step caused an unavoidable partial m1A → m6A conversion via a base-catalyzed Dimroth rearrangement [3,12]. The m1A and m6A species were separated using a Hewlett-Packard 1100 HPLC system with a Dionex DNA Pac PA-100 column and diode array detector. The mobile phase was made up of 40% 1.5 M ammonium acetate, 30% acetonitrile and 30% deionized water. Isocratic elution was performed at a flow rate of 10 mL/min. In addition to the m1A species, the m6A species including Tm6A-, Gm6A-, Am6A-, and Cm6A-oligo were also collected. NMR samples were prepared by dissolving 0.5 μmol of purified DNA samples into 500 μL of buffer solution containing 150 mM sodium chloride, 10 mM sodium phosphate (pH 7.0), and 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid.

2.3. NMR study

All NMR experiments were performed using Bruker ARX–500 and AV-500 spectrometers operating at 500.13 and 500.30 MHz, respectively. All experiments were acquired at 25°C unless stated otherwise. For studying labile proton signals, the samples were prepared in a 90% H2O/10% D2O buffer solution. One-dimensional (1D) imino spectra were acquired using the water suppression by gradient-tailored excitation (WATERGATE) pulse sequence [13,14]. Two-dimensional (2D) WATERGATE- nuclear Overhauser effect spectroscopy (NOESY) were performed with a mixing time of 300 ms. For melting studies, 1D WATERGATE experiments were performed from 25 to 95°C at a step of 2.5°C. In order to study the non-labile proton signals, the solvent was exchanged with a 100% D2O buffer solution. 2D NOESY experiments were performed with a mixing time of 300 ms and a data size of 4 K × 512. The acquired data were zero-filled to give 4 K × 4 K spectra with a cosine window function applied to both dimensions.

2.4. UV optical melting study

UV absorbance data at 260 nm were measured versus temperatures from 25 to 95°C at a heating rate of 0.8°C/min using a Hewlett-Packard 8453 Diode-Array UV–Vis spectrophotometer. The DNA sample concentration was kept at 3 μM with 150 mM sodium chloride and 10 mM sodium phosphate (pH 7.0) in 1 mL deionized water, and a 10 mm path length cuvette was used. Thermodynamic parameters were determined from the melting curves using the software MELTWIN version 3.5 (available from Jeffrey A. McDowell at www.meltwin.com).

3. Results and discussion

3.1. Influence of m6A on UV melting studies

To determine the influence resulting from the m1A → m6A conversion, we have performed UV melting studies of TA-, Tm1A- and Tm6A-oligo, starting from 25 to 95°C and then back to 25°C, at a step of 0.8°C and 1 min hold time at each temperature. Such a melting cycle, including both the heat up and cool down periods, took ~8 h. For TA- and Tm6A-oligo, both the melting temperatures (Tm) extracted from the heating and cooling curves were very similar (~0.1°C difference) whereas a significant difference of ~5°C was observed for those of Tm1A-oligo (Appendix A, S1). We believe this difference was due to the presence of m6A species resulted from Dimroth rearrangement in Tm1A-oligo at higher temperatures, switching T-m1A Hoogsteen base pair to T-m6A Watson–Crick base pair. To verify this, we desalted the sample after a UV melting cycle and performed HPLC analysis. The resulting chromatogram shows a total of ~50% of m1A was converted to m6A in a melting cycle (Appendix A, S2). For the m6A species, NMR analysis shows the T4 imino proton shifted downfield to ~14 ppm, suggesting T-m1A Hoogsteen base pair was switched to T-m6A Watson–Crick base pair when m1A was converted to m6A (Appendix A, S2).

3.2. Thermodynamics from NMR melting curves

In order to obtain reliable thermodynamic results, we attempted to use variable temperature 1D 1H NMR experiments to construct the melting curves based on proton chemical shift data. To validate this method, we measured the Tm values of TA-oligo using four well-resolved signals [10], namely, T12 methyl, T12 H6, T4 H6 and C13 H5. The Tm values were all found to be ~77–79°C, which agree well with the value of ~77°C as obtained from UV melting study (Appendix A, S1). The similar Tm values obtained from these various nucleotide positions also suggest the melting process is cooperative.

To determine the effect of m1A on the thermodynamics of dsDNA, we decided to construct the melting curves using the
chemical shifts of T12 methyl proton signals because (i) the methyl proton region was less crowded and (ii) the two resolved T12 methyl protons of the m1A and m6A species allow us to study the melting behavior of both species independently (Appendix A, S3). The \( T_m \) values and thermodynamic parameters of m1A and m6A species extracted from these melting curves (Appendix A, S4) are summarized in Table 1. Upon methylation at the N1 site of adenine, the \( T_m \) value was reduced by \( 10^\circ C \) but was reduced by only \( 3^\circ C \) for methylation at the N6 site. This demonstrates that m1A \( \rightarrow \) m6A conversion in Tm1A-oligo will affect the reliability of thermodynamics extracted from conventional melting methods.

### 3.3. Base pairing modes

Similar to our previous NMR findings in Tm1A-oligo [10], m1A also exhibited a local structural effect in Gm1A-, Am1A- and Cm1A-oligo. This was supported by the formation of stable flanking Watson–Crick base pairs which were evidenced by (i) G15 and G5 imino signals, (ii) G15 imino–C3 amino nuclear Overhauser effects (NOEs) and (iii) G5 imino–C13 amino NOEs (Appendix A, S5–S7). The proton chemical shifts of these oligomers are summarized in Appendix A, S8–S10.

### Table 1

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>( T_m, ^\circ C )</th>
<th>( \Delta H, \text{kcal mol}^{-1} )</th>
<th>( \Delta S, \text{cal K}^{-1} \text{mol}^{-1} )</th>
<th>( \Delta G_{37}, \text{kcal mol}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>78.5 (0.5)</td>
<td>(-54.2 (0.2))</td>
<td>(-154.2 (0.7))</td>
<td>(-6.4 (&lt;0.1))</td>
</tr>
<tr>
<td>m1A species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tm1A</td>
<td>68.3 (0.2)</td>
<td>(-52.1 (0.7))</td>
<td>(-152.5 (2.0))</td>
<td>(-4.8 (0.1))</td>
</tr>
<tr>
<td>Gm1A</td>
<td>68.5 (0.4)</td>
<td>(-50.0 (1.2))</td>
<td>(-146.3 (3.6))</td>
<td>(-4.6 (0.1))</td>
</tr>
<tr>
<td>Am1A</td>
<td>64.6 (0.5)</td>
<td>(-45.4 (0.6))</td>
<td>(-134.5 (2.0))</td>
<td>(-3.7 (&lt;0.1))</td>
</tr>
<tr>
<td>Cm1A</td>
<td>61.2 (0.2)</td>
<td>(-45.3 (1.1))</td>
<td>(-135.5 (3.3))</td>
<td>(-3.3 (0.1))</td>
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<tr>
<td>m6A species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tm6A</td>
<td>75.2 (0.3)</td>
<td>(-52.7 (1.9))</td>
<td>(-151.3 (5.7))</td>
<td>(-5.8 (0.2))</td>
</tr>
<tr>
<td>Gm6A</td>
<td>68.5 (0.5)</td>
<td>(-49.6 (1.6))</td>
<td>(-145.2 (4.8))</td>
<td>(-4.6 (0.1))</td>
</tr>
<tr>
<td>Am6A</td>
<td>64.6 (0.5)</td>
<td>(-48.5 (0.5))</td>
<td>(-143.5 (1.6))</td>
<td>(-4.0 (&lt;0.1))</td>
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<tr>
<td>Cm6A</td>
<td>60.8 (0.1)</td>
<td>(-41.8 (0.5))</td>
<td>(-125.2 (1.4))</td>
<td>(-3.0 (&lt;0.1))</td>
</tr>
</tbody>
</table>

* The results were based on fitting the melting curves (T12 methyl proton chemical shift versus temperature) using software MELTWIN 3.5. 1D NMR experiments were repeated three times for each sample from 25 to 95 \( ^\circ C \) at a step of 2.5 \( ^\circ C \). The average values were shown with the S.D. in parentheses.

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Fig. 2. (A) Proposed base pairing mode of G m1A in Gm1A-oligo. Observable NOEs were indicated by arrows. (B) These NOEs were found in the WATERGATE-NOESY spectrum at 0 \( ^\circ C \) and a mixing time of 300 ms. (C) The appearance of G4 H1, and m1A14 H62 and H61 in the 1D WATERGATE \(^1^H\) spectra at lower temperatures supports the proposed base pairing mode. (D) The glycosidic orientation of G4(anti) m1A14(anti) was evidenced by the relative intranucleotide NOE intensities.
3.3.1. G\textsubscript{m1A} base pair

In G\textsubscript{m1A}-oligo, we found that G/G\textsubscript{m1A} adopted a G(\textit{anti})/G\textsubscript{m1A} (\textit{syn}) base pairing mode involving two hydrogen bonds (Fig. 2A) as evidenced by the characteristic G4 H1–m1A14 H8 NOE (Fig. 2B, i), and the appearance of G4 imino and two m1A14 amino signals (Fig. 2C). The assignment of the two amino signals was based on their characteristic NOEs with m1A14 CH\textsubscript{3} (Fig. 2B, ii and iii), with the more downfield one being assigned to hydrogen-bonded m1A14 H62. Compared with C13 H5–H6 NOE, the H8–H1\textsubscript{0} NOE was much weaker in G4 but similar in m1A14, revealing the G4(\textit{anti}) and m1A14(\textit{syn}) glycosidic orientations (Fig. 2D) \[15\].

For this G(\textit{anti})-m1A(\textit{syn}) base pair, the expected G4 H1–m1A14 H62 NOE was not observed even at 0 °C. This was probably because G4 imino and m1A14 amino protons were much weaker than those of T4 and m1A14 in Tm1A-oligo (Appendix A, S5D), suggesting the hydrogen bonds in G(\textit{anti})-m1A(\textit{syn}) were weaker than those in T(\textit{anti}) m1A(\textit{syn}) Hoogsteen base pair. However, the observed destabilizing effect of G\textsubscript{m1A} and T\textsubscript{m1A} was similar, probably because the planar bicyclic ring of G in G(\textit{anti})-m1A(\textit{syn}) provides larger stacking surface and thus better stacking interactions than the monocyclic ring of T in T(\textit{anti})-m1A(\textit{syn}) \[16\].

3.3.2. A\textsubscript{m1A} base pair

A single hydrogen-bonded A(\textit{anti})-m1A(\textit{syn}) base pair was found to be in equilibrium between two conformations in Am1A-oligo (Fig. 3A). This was supported by the appearance of (i) m1A14 H62 which was assigned by m1A14 H62–CH\textsubscript{3} NOE (Fig. 3B), (ii) A4 H61 which was assigned by its NOEs with the neighboring C3 and C13 amino protons (Fig. 3C), and (iii) an NOE between A4 H2 and m1A14 H8 (Fig. 3D). NOEs were observed between A4 H61 and C3/C13 amino protons but not between m1A14 H62 and C3/C13 amino protons probably because the exchange rate between m1A14 H62 and water is much faster than that between A4 H61 and water. This is evidenced by the appearance of A4 H61 at 25 °C in the 1D WATERGATE spectrum but m1A14 H62 at 5 °C (Appendix A, S11).
The glycosidic A4(anti) and m1A14(syn) orientations were supported by the relative intensities of A4 and m1A14 H8–H1' NOEs (Fig. 3E). Since only one set of resonance signals was observed (Appendix A, S6), we believe the two A(anti) m1A(syn) conformations underwent rapid exchange and therefore the internucleotide A4 H2–m1A14 H62 and A4 H61–m1A14 H8 NOEs were not observed even at 0 °C. Owing to this conformational exchange process, the chemical shift of A4 H61 (~6.4 ppm) was less downfield than that of normal adenine bonded aminos (~7–8 ppm) [17] because the observed chemical shift was the weighted average of a bonded amino (Fig. 3A, right conformer) and a free amino (Fig. 3A, left conformer). Similarly, the chemical shift of m1A14 H62 (~8.7 ppm) was also affected by this conformational exchange process and thus being less downfield than that of m1A in T m1A Hoogsteen base pair (~9.5 ppm) and G-m1A mismatch (~10.1 ppm). The broadened A4 H2 peak at lower temperatures also supports the conformational exchange process (Appendix A, S6D). In fact, such exchange of single hydrogen-bonded conformations have also been characterized in a DNA double-helix containing an A:A mismatch [18], with both adenines adopting the anti glycosidic orientation. Unlike the pairing modes of T(anti)-m1A(syn) and G(anti)-m1A(syn) in which two hydrogen bonds are present, the single hydrogen-bonded base pairing mode in A(anti)-m1A(syn) probably accounts for the observed larger destabilizing effect in dsDNA.

3.3.3. Cm1A base pair

In Cm1A-oligo, C4 and m1A14 amino protons were not observed even at lower temperatures, suggesting no favorable pairing interactions were present between C4 and m1A14. Both C4 H6–H1' and m1A14 H8–H1' NOEs were found to be much weaker than C13 H5–H6 NOE, revealing C4 and m1A14 adopted the anti glycosidic orientation (Fig. 4A). Based on previous NMR and molecular dynamics results [19,20], two possible single hydrogen-bonded base pairing modes, although not very stable, have been proposed for C(anti)-A(anti) base pair (Fig. 4B). However, in Cm1A-oligo, such single hydrogen-bonded interactions between C and m1A seem to be unfavorable due to the steric effect of the methyl group at the N1 site of adenine. Thereby, it is not likely that favorable pairing interactions would exist in C(anti)-m1A(anti) base pair, which accounts for the observed largest destabilizing effect in dsDNA:

3.4. m1A and m6A on thermodynamics of dsDNA

From our thermodynamics results (Table 1), we observed a significant influence upon the rearrangement of the methyl group from N1 to N6 in T-m1A base pair. This can be rationalized by the fact that T-m1A adopts Hoogsteen whereas T-m6A adopts Watson–Crick base pairs. However, such influence was negligibly small in G-m1A, A-m1A and C-m1A base pairs. Based on our investigations on base pairing modes, it is probably that the rearrangement of the methyl group did not affect the pairings of G-m1A, A-m1A and C-m1A. On the other hand, it is also possible that the m6A species adopt different pairing modes which possess similar thermodynamic stabilities as the m1A species.

In summary, we have successfully used NMR spectroscopy to reliably determine the relative destabilizing effect of base pairs involving m1A on dsDNA, which follows the order: T-m1A > G-m1A > A-m1A > C-m1A. From our investigations, both T(anti)-m1A(syn) Hoogsteen and G(anti)-m1A(syn) base pairs contain two hydrogen bonds and their base pairing modes are similar. A(anti)-m1A(syn) can undergo rapid exchange between two single hydrogen-bonded conformations. No favorable pairing interactions have been observed in C(anti)-m1A(anti) base pair. These structural findings well rationalize the above destabilizing trend of m1A. In addition to the inherent sequence-dependent conformational flexibility in DNA [11], the formation of different base pairs at the methylation site also affects the energetics of base flipping. The present thermodynamic results provide us insights into the m1A flipping process in dsDNA and enhance our understandings of the mutagenicity of m1A in DNA replication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.017.

References