NMR studies on oligo-deoxyribonucleotides containing N^6-methyladenine

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500 MHz ^{1}H-NMR and NOE measurements of d(GGATCC) and d(GGm^{6}ATCC) show that both oligonucleotides assume a B-DNA conformation at low temperature. Around the melting temperature, however, the single and double strands of the *N*-methylated form are in slow exchange on the NMR time scale. The preferred conformation of the adenine methyl group, *cis* to N^1, retards base pairing and also destabilizes the double helix.

Synthetic oligonucleotide *NOE* *Recognition site* dam *methylase*

1. INTRODUCTION

Two types of methylation of DNA bases are known to occur [1], either at the C^5 position of cytosine or on the amino group of adenine. Both types influence restriction endonuclease interactions in prokaryotes [1,2]. A methylase specific for the sequence GATC (*dam* methylase) [3] appears to act with a certain lag period behind replication on newly synthetized DNA daughter strands which are transiently unmethylated [4]. The *dam* methylation may be also involved in strand discrimination during post-replication mismatch repair [5].

In N^6-methyladenine the methylamino group is coplanar with the purine ring and its rotation is hindered [6]. The *cis* form is strongly favoured [7] for which only Hoogsteen pairing would be possible. *N*-methylation has a destabilizing effect on polydeoxynucleotide duplexes [8] and decreases the rate of polI DNA polymerase reaction [9].

Thus, the question arises, whether or not N^6-methylation of adenosine produces a conformationally different form of DNA which would be recognized by an enzyme. We report here high resolution NMR studies on GGm^{6}ATCC and GGATCC as part of a programme to answer this question.

2. MATERIAL AND METHODS

2.1. *Synthesis of d(GGATCC) and d(GGm^{6}ATCC)*

The synthesis of the oligonucleotides was performed by the phosphotriester and phosphoamidite methods. The details of the chemical syntheses will be described elsewhere [10]. They may be outlined as follows: (a) Preparation of 5′-*O*-4,4′-dimethoxyltrityl-N^6-methyl-2′-deoxy-adenosine. (b) Preparation of the protected nucleotide monomers, 3′-*O*-*m*-chlorophenyl-phosphodiester and 3′-*O*-morpholinomethoxyphosphine derivatives. (c) Assembly on silica gel support by the phosphotriester and phosphoamidite procedures. (d) Deprotection and purification by high-performance liquid chromatography (HPLC).

2.2. *Preparation of solutions*

The oligonucleotides were dissolved in D_2O, 150 mM NaCl, 10 mM phosphate buffer (pH 7.2) and 0.2 mM EDTA. The strand concentration was 2 mM. Chemical shifts reported are relative to 4,4-dimethyl silapentane-1-sulphonate.

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2.3. *NMR spectra*

NMR spectra were recorded on a Bruker WM500 spectrometer. The NOEs were observed by cycling 32 transients on resonance, followed by 32 transients off resonance. Irradiation was applied for 100 ms (above which spin diffusion effects were visible) at a power level which gives 90% saturation of the irradiated resonance. The estimated error in NOE magnitudes is $<0.5\%$. For the measurements of exchangeable protons the same buffer solution was used in 90% H_2O/10% D_2O. A time-shared pulse sequence, $\theta_x - \tau - 8_{-x}$ [11] was used. The carrier was placed on the water resonance and a delay time, τ, of 350 μs inserted between two pulses.

3. RESULTS AND DISCUSSION

Fig.1 shows the proton spectrum of the methylated hexamer as a function of temperature. Below 40°C two resonances were observed for each proton, corresponding to the single and double strands. To our knowledge, this is the first case of slow exchange (on an NMR time scale) for the single to double strand transition in a DNA. Nevertheless, during the melting process the resonances for both the single and double strands are broadened by exchange. For example, at 32°C – which corresponds to the T_m – the thymine methyl resonance of the double strand has a line width of 20 Hz whereas it drops to 7 Hz at 4°C. The excess line width gives a measure of the life time of the double strand as

$$\pi\Delta\nu_{1/2}\ (\text{obs}) = T_2^{-1} + t_{\text{exch}}^{-1}$$

where T_2 is the spin-spin relaxation time in the absence of exchange ant t the lifetime of the species. Thus the lifetime of the double helix is ~25 ms at 32°C.

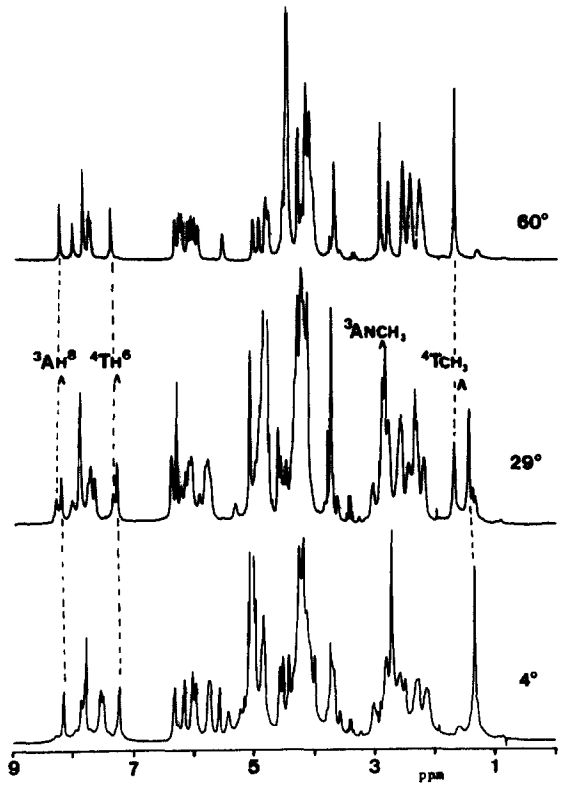

Fig. 1. 500 MHz spectrum of GGm^{6}ATCC at 4, 29 and 60°C. Note the duplication of all resonances at 29°C. Connections for ^{3}AH^8, ^{4}TH^6 and ^{4}TCH^3 only have been shown for the sake of clarity.

For the non-methylated oligonucleotide d (GGATCC) we observe a T_m of 45°C and very little broadening of the resonances at this temperature relative to those observed at low temperature where the single strand is absent.

The base and anomeric protons can be easily assigned by standard techniques [12] which rely heavily upon the use of pre-steady state nuclear Overhauser enhancements (NOEs). The techniques, however, require an assumption about the form of the helix.

Spectra obtained in 90% H_2O at 4°C show 3 resonances in the range 12.5–14 ppm. Thymine imino protons generally resonate at lower field than the guanine imino protons. Irradiation of the lowest field resonance gives a large NOE to the resonance at 7.80 ppm and significantly no NOE to the adenine methyl group. This proton at 7.80 ppm shows the longest T_1 of all the base protons and does not exchange with D_2O at 90°C over 3 h. The observed NOE is thus to the adenine H^2. The base pairing for the m^{6}A-T must be Watson-Crick and the adenine methyl group must therefore be *trans* relative to N^1.

Further confirmation of the form of the helix is given by NOEs observed on irradiation of the purine H^8 and pyrimidine H^6 protons (fig.2). In a right-handed B form helix NOEs should be observed to the anomeric proton of its own sugar residue (3.7–3.8 Å) and to the anomeric proton of

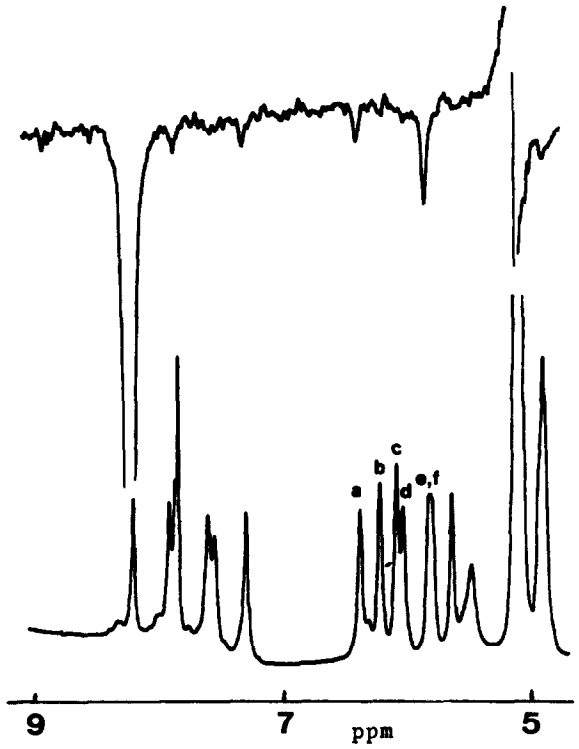

Fig. 2. Partial 500 MHz NMR spectrum of GGm^{6}ATCC and NOE spectrum irradiating ^{3}A(H^8). Sugar resonances are lettered as in table 1.

the sugar in the 5′-direction (3.5–3.6 Å) [12]. In a left-handed B form helix a similar intra-residue NOE would be observed. The anomeric proton of the residue in the 5′-direction, however, is too far (>5 Å) for any NOE to be transmitted, whereas the anomeric proton of the residue in the 3′ direction is close (3.8–4.0 Å). Thus, in the right-handed helix irradiation of each cytidine H^6 should give rise to two NOEs on anomeric protons (^{4}T and ^{5}C, ^{5}C and ^{6}C). In a left-handed helix irradiation of ^{5}C(H^6) would give two anomeric NOEs (^{5}C and ^{6}C) whereas irradiation of ^{6}C(H^6) would only give an NOE to its own anomeric proton. The two C(H^6) proton resonances are readily identified in resolution enhanced spectra by their coupling with C(H^5). Irradiation of each one gives rise to two anomeric NOEs (see table 1). Upon irradiation of T(CH$_3$) at 1.36 ppm two NOEs are observed in the aromatic region, 11% on the resonance at 7.24 ppm and 5% on that at 8.17 ppm. The larger NOE must be to T(H^6), the smaller one an inter-residue

Table 1

Pre-steady-state NOEs (%) (0.1 s pre-irradiation time) observed on H$_1^1$ protons upon irradiation of some of the base protons of d(GGm^{6}ATCC) at 4°C

Resonance	a	b	c	d	e*	f*
	(^{3}A)	(^{6}C)	(^{5}C)	(^{4}T)	(^{2}G)	(^{1}G)
^{3}A(H^8)	1.9				5.7	
^{2}G(H^8)(+)					~3.5	~3
^{1}G(H^8((+)						~2.5
^{5}C(H^6)			2.1	3.1		
^{6}C(H^6)		1.8	2.2			
^{4}T(H^6)	3.4			2.1		

* The assignments of the guanosine H^8 and H$_1^1$ protons are not certain, since the anomeric proton resonances are almost coincident

NOE to A(H^8). There is no ambigiuity as A(H^2) has already been identified. Irradiation of these two base resonances gives rise to two anomeric NOEs (table 1). Therefore, there is no change in the helix sense in the fragment ^{2}G–^{6}C; if a right- to left-handed junction existed, irradiation of ^{3}A(H^8) or ^{4}T(H^6) would give an NOE only to its own anomeric protons. The assignment of the anomeric protons is derived from tracing the path of the common NOEs observed from irradiation of base protons on adjacent residues [12]. The two guanosine H$_1^1$ protons resonances are unfortunately almost coincident.

The small values of the observed NOEs also show that all the bases are in the *anti* conformation. If any were in the *syn* conformation the H^6–H$_1^1$ or H^8–H$_1^1$ distance would be reduced to about 2.2 Å, in which case an NOE of ~35% would be expected. This also confirms the absence of Hoogsteen pairing. The same procedure has been followed for the unmethylated oligomer. The absolute values of the NOEs observed to the anomeric protons are all within ±1% of those reported for the methylated oligomer, showing that at most minor differences exist between the two oligomers at low temperature.

Thus methylation on both strands in the sequence GATC produces a destabilization of the helix and a very much slower exchange between single strand and duplex, without any major changes in the helix geometry from a normal B form.

For exchange between two species the appearance of separate resonances for each species or a single averaged resonance is determined by the product of the lifetime of the species and the separation (in Hz) of the resonances of a particular proton in the two environments. For the $T(CH_3)$ resonance the difference is 120 Hz for the methylated oligomer and 110 Hz for the unmethylated one. Only small differences are observed for corresponding protons in the two oligomers and thus the observation of slow exchange must be due to a very different lifetime for either (or both) the single and the double strand, compared to the unmethylated oligomer.

A similarly decreased exchange rate is also observed for the hemimethylated $GGm^6ATATCC$ sequence [13].

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