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An NMR study of A-T base pair opening rates in oligonucleotides. Influence of sequence and of adenine methylation

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ABSTRACT We report relaxation time measurements by semi-selective and totally selective NMR techniques on the thymidine imino protons of d(GGATATCC) and d(GGm^{6}ATATCC). For these oligonucleotides helix fraying, rather than single base pair opening, is the major exchange mechanism even 25°C below the Tm. We have therefore applied a new saturation transfer technique to measure exchange rates at temperatures where fraying has a very small or negligible contribution. Measurements of exchange rates as a function of temperature give significantly different activation energies for base pairs 3 and 4 in d(GGATATCC). Adenine methylation results in a slowing down of the opening rate for the m^{6}A-T base pair but surprisingly has an even greater effect upon the adjacent non-methylated A-T base pair.

INTRODUCTION

NMR measurements of the relaxation rates of the imino protons in polynucleotides or in DNA oligomers can provide information on base pair opening rates and, from variable temperature studies of these, activation energies. A *priori* the absolute rate of opening of a base pair and the corresponding energy of activation could be sequence dependent even if the base pair opens without opening of one or both of the neighbouring base pairs. In particular there have been many studies reported on the dynamics of A-T base pairs in different environments (1-16).

Activation energies for single base pair opening of 14 to 34 kcal/mole (3-7,10,12,15,16) have been reported. One study found that the activation energy for opening of a A-T base pair is sequence independent (3) while another found significant differences for the AATT or TATA core of dodecanucleotides.

In addition to the widely varying activation energies reported there is also the question as to whether absolute opening rates are sequence dependent. Rates differing by a factor of about two have been observed for A-T base pairs in the Pribnow sequence (6) compared with earlier reports.

At low temperature the relaxation rate of an imino proton is

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dominated by dipole-dipole relaxation and at a suitably high temperature by exchange. Both mecanisms will always contribute, to a varying degree, to the observed relaxation and their separation is not straight forward. In addition, care must be taken that proton exchange with solvent is a real measure of the base pair opening rate. In other words, proton exchange must occur each time a base pair opens, the open limited state (14,18,19). Finally, the observed relaxation rates can be a function of the NMR technique used to measure them. Totally selective, semi-selective or non-selective inversion may change the observed relaxation rate and in some cases non-single exponential decay is observed (10).

In order to examine further the dynamics of A-T base pairs we have studied two oligonucleotides d(GGATATCC) and d(GGm^{6}ATATCC) by several techniques including a new method which we have recently developped (19) which avoids the complication of estimating the dipolar contribution to the observed relaxation. We have previously observed (20-22) that adenine methylation considerably slows down the rate of helix formation and dissociation.

MATERIALS AND METHODS

d(GGATATCC) and d(GGm^{6}ATATCC) were synthesized by a modified phosphate triester and phosphoamidite method on silica gel support (A. Guy, L. Wagrez, D. Molko and R. Téoule, unpublished results). The oligonucleotides were 3 mM in strand dissolved in 90% H_2O/10% D_2O and 150 mM NaCl. The pH and phosphate concentration are given in the text.

NMR spectra were recorded at 500 MHz on a Bruker WM-500 spectrometer. Semi-selective T_1 measurements were made using a hard pulse 1-τ-4-τ-6-τ-4-τ-1 sequence (14641) to suppress the solvent resonance with the carrier placed in the center of the imino proton region. Selective T_1 measurements were made with the sequence

$$(\pi_1\text{-}n/80)_{80} - t\ (\pi/16\text{-}\tau_2\text{-}3\pi/16\text{-}\tau_2\text{-}3\pi/16\text{-}\tau_2\text{-}\pi/16)$$

For the inverting DANTE pulse τ_1=185 μsec. The 1331 hard pulse, with τ_2=88 μsec was used to attenuate any residual water resonance. The transmitter was attenuated by 10 db to give a $\pi/80$ pulse of 1.5 μsec. The same 1331 pulse sequence was used to measure the imino resonance magnetization in the absence of solvent saturation.

The imino resonances were assigned by standard inter-residue NOE measurements.

RESULTS AND DISCUSSION

We first used a semi-selective inversion method to measure the relaxation rate of one of the two A-T imino protons in d(GGATATCC). The 14641 pulse sequence inverts simultaneously all the imino resonances while those in the aromatic region (which includes the neighbouring adenosine H^2 as well as all the amino resonances) are very much less affected. The final spectrum shows intensities of less than 6% for these protons relative to the imino protons.

As relaxation rates can give exchange lifetimes only in the open limited case we first measured these rates at pH 7.0 in the presence of 10 mM phosphate as catalyst. Both the pH and phosphate concentration were then increased until further addition did not influence the observed relaxation rates. As exchange is base catalysed and further addition of base has no effect, the final conditions, 50 mM phosphate pH 8.0, correspond to an open limited situation.

The observed relaxation rates as a function of temperature are shown in Fig.1 for the thymidine imino proton of base pair 3. The relaxation rate decreases on raising the temperature from 0°C and reaches a minimum at ca. 8°C. Above this temperature the relaxation rate increases again. Measurements were made up to 36°C above which temperature exchange broadening makes further measurements impossible. From previous studies of the non exchangeable protons (22) the Tm of the oligonucleotide was found to be 51°C.

In the high temperature region, 28 to 36°C, the observed relaxation rate changes linearly with reciprocal temperature. As we would expect exchange to dominate relaxation and the dipolar contribution to be fairly constant over this narrow temperature range, the slope should give an estimate of the activation energy for exchange. A value of 45 Kcal/mole was found and correcting for the dipolar contribution could only increase this value as the latter would be greater at 28°C than at 36°C. The observed relaxation rates derived from the line widths of the imino resonance are also plotted in Fig.1 and these show the same behaviour as a function of temperature.

The only explanation for such a high activation energy is that exchange occurs via a pathway involving the opening of base pairs 1, 2 and 3. This phenomenon, fraying of the ends of the helix, has been observed several times (1,16,17) giving rise to anomalously high activation energies. However, in these cases the helix was terminated by two or more

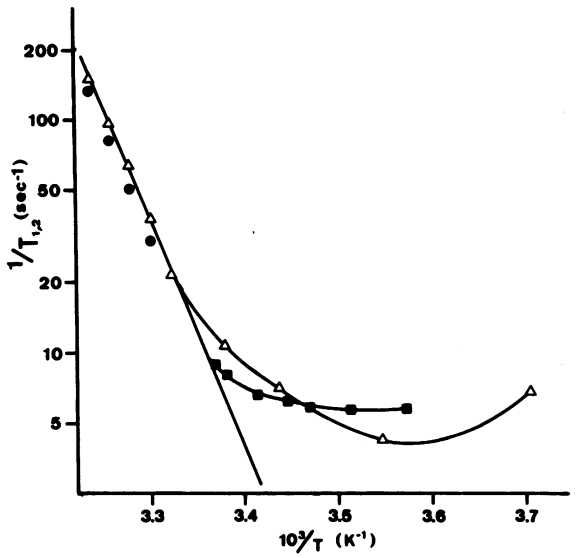

Figure 1: Arrhenius plots of observed relaxation rates of the ^{3}A-T imino proton of d(GGATATCC): (△) by semi-selective inversion method, (◐) by selective inversion method (19) and (●) derived from the line widths.

A-T (or A-U) base pairs, whereas the two G-C base pairs on either end of this oligonucleotide could be expected to inhibit fraying even though the terminal base pair should exchange faster than its neighbour. It is therefore necessary to measure the exchange contribution to relaxation at lower temperature where single base pair opening may dominate exchange.

If the water resonance is saturated continuously, i.e. $dM_z(H_2O)/dt=0$, the imino proton relaxation rate is described (19) by

$$\frac{1}{T_1{}'_A} = \frac{M^A{}_0}{M^A{}_0 - \bar{M}^A{}_0} \cdot \frac{1}{\tau_A} \qquad (1)$$

where $M^A{}_0$ and $\bar{M}^A{}_0$ are the equilibrium magnetizations of the imino proton without and with solvent saturation, respectively and τ_A is the exchange lifetime of proton A. The dipolar contribution to the observed relaxation, $1/T_{1A}$, can also be found from

$$\frac{1}{T_{1A}} = \frac{M^A{}_0 \cdot T_1{}'_A}{\bar{M}^A{}_0} \qquad (2)$$

Using a highly selective DANTE sequence (23) to invert the imino resonance we have measured the relaxation rate in the temperature range 7 to 24°C, Fig.1. At all temperatures single exponential decay was observed. In the lower part of this temperature region the observed relaxation rates were faster than those observed by the semi-selective pulse method. This

Table I: Temperature dependence of lifetimes of A-T base pairs (T_1 measurements)

d(GGATATC):		^{3}A-T		^{4}A-T	
	t°C	% sat.	τ (msec)	% sat.	τ (msec)
	15.4	0.62	277	0.58	297
	17.2	0.72	222	0.53	200
	20.0	0.77	200	0.84	153
	22.8	0.87	153	-	-
	23.9	0.87	135	0.84	96
d(GGm^{6}ATATCC):		^{3}m^{6}A-T		^{4}A-T	
	t°C	% sat.	τ (msec)	% sat.	τ (msec)
	18.3	0.48	435	0.29	943
	20.0	0.58	339	0.42	855
	21.2	0.54	400	0.345	654
	22.5	0.66	282	0.45	714
	23.5	0.71	238	0.55	505
	24.8	0.73	229	0.56	523

is in agreement with results on poly d(A-T) (10). If neighbouring protons (in this case the G-C imino of base pair 2, the A-T imino of base pair 4, the adenosine Watson-Crick amino proton and the adenosine H^2) are touched by the inverting pulse this group of protons will tend to relax together following zero-quantum transfer of magnetization. Above 15°C the selective rates are slower than the semi-selective rates. While we cannot explain this quantitatively, we note that two of the four neighbouring protons now relax faster than the observed imino proton. The adjacent G-C imino proton shows more rapid exchange with solvent, but particulary the adenosine amino group which relaxes very rapidly via rotation about the C^6-amino bond as we have previously observed in d(GGATCC) (21).

We have measured the selective relaxation rates of the two thymidine imino protons in d(GGATATCC) and also the transfer of magnetization resulting from saturation of the water resonance as a function of temperature. These results are given in Table I for the temperature range 15 to 25°C. Measurements obtained below 15°C have not been used to evaluate the imino proton exchange rate. At low temperature we observe an additional effect upon solvent saturation - that of a decrease in the intensity of resonances of non-exchangeable protons exposed to the solvent. This can be explained by a negative NOE from DNA bound water molecules. The effect upon the imino resonances would be somewhat less, arising only from spin diffusion from the adenosine H^2 or amino

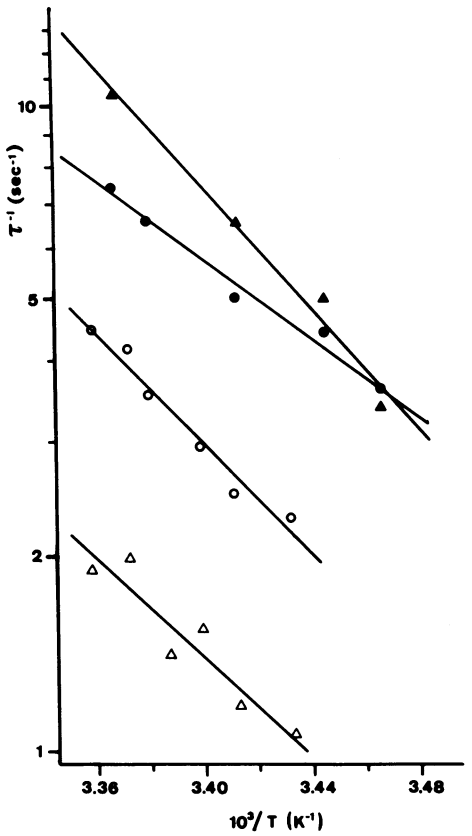

Figure 2: Arrhenius plots of inverse exchange lifetimes of imino protons of d(GGATATCC): (●) ^{3}A-T and (▲) ^{4}A-T, and of d(GGm^{6}ATATCC): (○) ^{3}m^{6}A-T and (△) ^{4}A-T.

protons, but an uncertainty remains and we have therefore not used the low temperature results. From eq.(1) we have calculated the exchange lifetimes and these are shown in the form of an Arrhenius plot in Fig.2. In the region 20 to 25°C the imino proton of base pair 4 exchanges more rapidly with solvent than that of base pair 3. Helix fraying can not be a major factor in this region as this would produce the reverse order of exchange lifetimes. The difference observed for the slopes for the two imino protons is quite striking. For base pair 3 the slope corresponds to an activation energy of 13.5 kcal/mole while for base pair 4 it corresponds to 21.5 kcal/mole. This difference is very much larger than any possible experimental error and shows a strong effect of the neighbouring base pairs upon the activation energy. In the Pribnow sequence differences of less than 20% were observed for different A-T base pairs (6).

In our studies on the dam methylase site GATC (20-22) we have observed that helix formation was slow on a proton NMR time scale. We have thus applied the same selective inversion technique to d(GGm^{6}ATATCC) to examine the effect of adenine methylation upon base pair opening. The results are shown in Table I and Fig. 2.

Imino proton exchange for the methylated base pair 3 is slowed down relative to the same base pair in d(GGATATCC) by a factor of about two. This could be easly explained by the increased basicity of the adenosine amino proton upon methylation resulting in stronger hydrogen bonding. In addition methylation increases the activation energy for exchange from 13.5 to 19.5 kcal/mole. Surprisingly, the most pronounced effect of adenine methylation is not upon the methylated base pair but on the neighbouring A-T base pair. The neighbouring G-C base pair seems to be very little affected in terms of exchange with solvent (results not shown).

Imino protons exchange for base pair 4 is slowed down by a factor of about six upon methylation of base pair 3. In addition the activation energy for exchange decreases from 21.5 kcal/mole to 17.5 kcal/mole. Clearly, adenine methylation strongly influences the neighbouring A-T base pair although the conformation of the oligonucleotide is not modified (22).

It has been suggested by Calladine (24) and demonstrated by Dickerson (25) that in a purine-pyrimidine sequence the positive propeller twist within a base pair in B DNA will give rise to a steric clash in the large groove. A similar clash in the small groove occurs in pyrimidine-purine sequences due to the negative propeller twist. In the methylated octamer purine-pyrimidine step between base pairs 3 and 4 the propeller-twist clash in the large groove will increase due to the presence to the N^6-methyl group in base pair 3. The isomerization of the methylamine group from the cis to the trans conformation (20-22, 26) will even further increase this clash, reducing the possibility of opening of base pair 4 even more, thus explaining the dramatic slowing down of the imino proton exchange of base pair 4. The negative propeller twist between the two central base pairs 4, on the other hand, should increase the exchange rates and decrease the activation energy, as observed. The subtle interplay between steric contacts on the edges of the base pairs will thus give rise to opposing phenomena and seemingly condradictory results.

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