The alternating conformation of analogues of poly[d(AT)]

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Abstract

Synthetic DNAs were prepared containing 6-methyl adenine (6-A) in place of adenine and 5-ethyl uracil (Et-U) or 5-methoxymethyl uracil (Mm-U) in place of thymine. All three modifications destabilized duplex DNAs to varying degrees. The binding of ethidium was studied to analogues of poly[d(AT)]. There was no evidence of cooperative binding and the "neighbour exclusion rule" was obeyed in all cases although the binding constant to poly[d(mAT)] was approximately 6 fold higher than to poly[d(AT)]. 31P NMR spectra were recorded in increasing concentrations of CsF. Poly[d(AEt-U)] showed two well-resolved signals separated by 0.55 ppm in 1 M CsF compared to 0.32 ppm for poly[d(AT)] under identical conditions. In contrast, poly[d(AmM-U)] and poly[d(mAT)] showed two signals separated by 0.28 ppm and 0.15 ppm respectively, only when the concentration of CsF was raised to 2 M. The signals for poly[d(AT)] in 2 M CsF were better resolved and were separated by 0.41 ppm. These results suggest that minor modifications to the bases may have conformational effects which could be recognized by DNA-binding proteins.

Introduction

The alternating conformation of DNA was proposed by Klug et al., (1) mainly on the basis of DNase I digestion patterns (2). These studies showed that in alternating poly[d(AT)] the ApT step adopted a different conformation from the TpA step. Although this is difficult to visualize without models, it was suggested that this difference was due to increased stacking of T residues on the A residues below them, with a concomittant decrease in stacking with the A residues above. This gives rise to an alternating conformation for the DNA backbone because the repeat unit of the helix is a dinucleotide. It seems likely that the 5-methyl group of T plays an important role. In the alternating conformation the methyl group is positioned above the five-membered ring of the A residues, thereby increasing the favourable stacking interactions.

These ideas have been confirmed and extended by 31P NMR spectroscopy (3,4,5). Since the phosphorous atoms in the ApT and TpA steps have slightly

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Figure 1. Structures of the base analogues used in this work. Mm^5U = 5-methoxymethyl Uracil. Et^5U = 5-Ethyl Uracil. m^6A = N^6-methyl Adenine.

different environments they give rise to two signals in the NMR of poly[d(AT)] rather than the single broad peak which is observed for calf thymus DNA. Similarly, two phosphorous signals are observed for poly[d(ABr^5U)], poly[d(Gm^5C)] and poly[rG-dm^5C] again implying that they adopt an alternating conformation (4,5,6). The separation of these signals can be increased at high ionic strength, especially in the presence of CsF (7). Of particular interest to the present study is the observation that poly[d(AU)] only adopts the alternating conformation at very high ionic strengths confirming the importance of the 5-methyl group of T (3).

Here we report the effects of substitution of 5-ethyl and 5-methoxymethyl pyrimidines (Et^5U and Mm^5U) as well as 6-methyl adenine (m^6A) on the alternating conformation of poly[d(AT)] (Figure 1). 5-Methoxymethyl-2'-deoxyuridine is a selective antiherpes agent (8,9), which is due to its phosphorylation by the herpes-induced thymidine kinase (10). Also the triphosphate inhibits preferentially viral DNA polymerase. At present it is not known whether it is incorporated into the DNA. The analogue, 5-ethyl-2'-deoxyuridine exerts an antiviral effect against vaccinia and herpes viruses (11,12) and has been shown to be incorporated into phage DNA (13) and lymphocyte DNA (14). 6-Methyl adenine (m^6A) is involved in many restriction-modification systems. For example, when m^6A is present, a restriction enzyme such as EcoRI, no longer cuts at the restriction site. Therefore, it is important to understand whether recognition of these unusual bases in duplex DNA may involve recognition of the modification per se or recognition of a different conformation induced by the modification. These recognition processes have been discussed by Drew and Travers (15).

Materials and Methods

Deoxynucleotide triphosphates: 5-Ethyl-2'-deoxyuridine was a gift from Dr. D. Ilse, Ortho Pharmaceuticals, Canada, Ltd., and 5-methoxymethyl-
2'-deoxyuridine was synthesized as described previously (9). The corresponding triphosphates (dET5UTP and dMM5UTP) were synthesized by using published procedures (16,17). dM5ATP was prepared from dATP by the Dimroth rearrangement (18). Other triphosphates were purchased from P-L Biochemicals.

**Duplex DNAs:** Poly[d(dAT)] and other synthetic DNAs were synthesized as described previously (19). The analogues were produced in a similar fashion except that dm5ATP and dET5UTP or dMM5UTP were included instead of dATP or dTTP as appropriate. In general, the analogues were added at one half the usual concentration (i.e. 1 mM dXTP instead of 2 mM) to conserve these resources but this reduction had only a minor effect, if any, on the final yield.

For NMR experiments, after purification and concentration, the DNAs were subjected to extensive sonication to reduce the molecular weight to below 200 base-pairs. The micro-probe of a Sonifier cell disrupter model W185 (Ultrasonics Inc.) was placed into the DNA at a concentration of 1 mg/ml. Ten second bursts with ten seconds rest was continued at maximum power (50 watts) for 20 minutes at 0°C. Finally, the DNAs were centrifuged to remove small particles before NMR analysis.

**Thermal denaturation profiles:** Tm's were measured in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA and 5 mM NaCl as described previously (19). A DNA of known Tm was always melted simultaneously to act as a control.

**Binding of Ethidium Bromide:** In the presence of duplex DNA the fluorescence of ethidium (excitation 525 nm, emission 600 nm) is enhanced approximately 30-fold (19). The initial buffer (2 ml) was 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 5 mM NaCl and 0.1 μg/ml ethidium bromide. The fluorescence (arbitrary units) was measured with a Turner spectrofluorometer upon the sequential addition of 1-10 μl aliquots of DNA at approximately 75 μM (concentration in base-pairs). It was assumed that poly[d(dAT)] and all analogues had extinction coefficients of 6,600 M⁻¹. From the increase in fluorescence upon addition of DNA a binding curve was constructed as described previously (20). This was analysed by Scatchard plots according to the theory of McGhee and von Hippel (21) with the aid of a least squares curve fitting computer program (22). In this way "K" (the intrinsic association constant) and "n" (the number of base-pairs occluded by the binding of one drug molecule) were elucidated.

**31P NMR:** Spectra were obtained on a Bruker AM300 NMR spectrometer operating at 121.5 MHz, using deuterium locking and broad-band proton
decoupling. The temperature was 25 ± 0.1°C. Typically 2K data points were collected, with a spectral width of 4800 Hz. Transients were accumulated until the signal-to-noise ratio was judged acceptable, between 10,000 and 60,000. Chemical shifts were measured using a capillary containing 5 mM trimethyl phosphate (TMP), and are quoted (after susceptibility corrections) in ppm relative to internal TMP. Positive shifts are to high frequency.

Samples were prepared as follows: To 1.0 - 1.5 ml. of polymer (see above) was added sufficient 0.1 M NaCl in D₂O to bring the total volume to 2.9 ml. After recording the spectrum, concentrated CsF solution was added to give an overall concentration of 1.0 M, 2.0 M or 3.0 M and the accumulation repeated.

### Results

In order to characterize the DNA analogues containing m⁶A, Et⁵U and Mm⁵U, Tms were measured, and these are detailed in Table I. The Tms of poly[d(AT)], poly[d(m⁶AT)] and poly[d(AEt⁵U)] agree with previously reported values (18,23). In general, each of the three substitutions leads to a decrease in the Tm though the effect is very variable depending on the sequence of the polymer. For example, the analogues of poly[d(TC)].poly[d(GA)] all have Tms about 11°C below that of the parent DNA. In contrast, for poly[d(AT)], substitution with m⁶A leads to a much larger decrease than Et⁵U which in turn leads to a much larger decrease than substitution with Mm⁵U. On the other hand the Mm⁵U analogues of poly[d(TG)].poly[d(CA)] and poly[d(ATC)].poly[d(GAT)] have Tms similar to those of the parent DNAs. All attempts to replicate duplex poly(dA).poly(dT)

<table>
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<th>Table I. Tms of synthetic polymers containing unusual bases</th>
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<tr>
<td>poly[d(AT)]</td>
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<tr>
<td>poly[d(TC)].poly[d(GA)]</td>
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<tr>
<td>poly[d(TC)].poly[d(CA)]</td>
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<tr>
<td>poly[d(ATC)].poly[d(GAT)]</td>
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<tr>
<td>poly[d(TTG)].poly[d(CAA)]</td>
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<tr>
<td>poly[dA].poly[dT]</td>
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in the presence of all three substituted triphosphates failed, as did the replication of poly[d(TTG)].poly[d(CAA)] with d\text{m}_{6}\text{ATP}.

Certain structural features of DNA can also be studied through drug binding. For example, propidium diiodide (a phenanthridinium drug related to ethidium bromide) binds to poly(dA).poly(dT) (but not to poly[d(AT)]) in a cooperative manner (24). Also ethidium will only bind to the 'Z'-conformation by inducing a transition from the left to the right-handed conformation at the intercalation site. This results in a distinctive binding isotherm (25). Scatchard plots for the binding of ethidium to poly[d(AT)] and 5-substituted pyrimidine analogues are shown in Figure 2. The data points are all well-fitted to the theoretical curves based upon the binding isotherm of McGhee and von Hippel (21). Thus the interactions are non-cooperative and values of \( n \) approaching 2 base-pairs are consistent with the neighbour exclusion model for ethidium intercalation. Also the binding constants (e.g. \( K = 4.3 \times 10^6 \text{M}^{-1} \) for poly[d(AT)]) are all similar suggesting that in the intercalated state ethidium does not interact with the 5 position of
pyrimidines. In contrast, the binding constant to poly[d(m\textsuperscript{6}AT)] is an order of magnitude higher (K = 27 x 10\textsuperscript{6} M\textsuperscript{-1}) although the value of n is still nearly 2 base-pairs (Figure 3). Presumably, the methyl groups in the 6 position provide additional stacking interactions for ethidium in the intercalated state.

The \(^{31}\)P NMR spectrum of poly[d(AT)] as a function of increasing concentrations of CsF is shown in Figure 4. These results agree well with those of previous authors (3) and provide good evidence for an alternating conformation which is enhanced as the ionic strength increases. The corresponding spectra for poly[d(AEt\textsuperscript{5}U)], poly[d(AMm\textsuperscript{5}U)] and poly[d(m\textsuperscript{6}AT)] are shown in Figure 5, 6 and 7 respectively. The observed chemical shift differences are listed in Table 2.

It can be seen that each polymer behaves differently. For poly[d(AEt\textsuperscript{5}U)] in 0.1 M NaCl, a shoulder is observed which becomes two well-defined peaks on

Figure 4. \(^{31}\)P NMR spectra of poly[d(AT)] in low salt buffer plus (a) 0M CsF, (b) 1M CsF, (c) 2M CsF.
addition of 1 M CsF. The separation between the signals is larger than for poly[d(AT)] at all concentrations of CsF. This suggests that poly[d(AEt^5U)] adopts a more alternating conformation than the parent polymer. On the other hand, poly[d(AMm^5U)], shows a shoulder in 1 M CsF and two signals only become apparent in 2 M CsF. Even here the separation is rather small (0.28 ppm) suggesting that poly[d(AMm^5U)] is rather reluctant to adopt the alternating conformation. Finally, poly[d(m^6AT)] is even more reluctant to change conformation. Two signals can barely be resolved in 2M CsF although in 3M CsF the separation is greater than that shown by poly[d(AMm^5U)].
The binding of ethidium to the analogues of poly[d(AT)] showed no unusual features; namely, there is no suggestion of cooperativity and in any event the concentration of ethidium (0.3 μM) is much lower than that required to bind to "Z" DNA (20 μM) (25). This indicates that these DNAs adopted an orthodox right-handed conformation. The binding constant to poly[d(m^6AT)] was about 6 times greater than the others; this is probably due to increased stacking interactions between ethidium and the additional methyl groups of the adenine residues. Crystallographic studies of intercalation complexes of ethidium (26) show that the methyl group of m^6A could interact favourably 

Table II. Chemical shift differences of the 31P NMR signals as a function of CsF concentration

<table>
<thead>
<tr>
<th>CsF (M)</th>
<th>Poly[d(AT)]</th>
<th>Poly[d(AEt^5U)]</th>
<th>Poly[d(AMm^5U)]</th>
<th>Poly[d(m^6AT)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Shoulder</td>
<td>Shoulder</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0.32</td>
<td>0.55</td>
<td>Shoulder</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.41</td>
<td>0.68</td>
<td>0.28</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>not determined</td>
<td>0.65</td>
<td>0.25</td>
<td>0.32</td>
</tr>
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</table>
with an intercalated ethidium. On the other hand, substituents on the 5 position of pyrimidines point away from the intercalation site so that an interaction seems unlikely.

While the binding of ethidium is relatively insensitive to small structural changes, the NMR signal of the phosphodiester backbone is not. As shown in Table 2, the chemical shift behaviour of poly[d(AT)] and its analogues are all different. Based on the theory of Klug et al., (1) we propose that the increased splitting shown by poly[d(A\(5^U\))] is due to even more pronounced stacking of \(5^U\) on A compared to T on A. On the other hand the reluctance of poly[d(A\(5^U\))] to adopt an alternating conformation may be due to the more hydrophilic nature of the methoxymethyl group which may have a greater tendency for solvent exposure rather than stacking. In the case of poly[d(m\(6^A\))], the preference for the non-alternating conformation is difficult to rationalize in terms of base stacking. It seems likely that some other determinant of conformation such as hydration may be involved.

These results highlight the fact that minor molecular replacements can have large conformational implications. Although NMR studies were only performed on analogues of poly[d(AT)] the variability in the Tms of other sequences containing the analogues (Table 1) suggests that the conformational consequences of these modifications are sequence dependent. Recently, we described a monoclonal antibody which recognized the alternating conformation of DNA (27). It seems likely that regulatory proteins and other DNA-binding proteins may also make use of this conformational recognition as well as direct recognition of the modification. Thus some of the antiviral activity of the deoxynucleosides of \(5^U\) and \(5^U\) may be due to detrimental alteration of the DNA conformation after incorporation of the analogues. Indeed for several 5-substituted deoxyuridine derivatives, it has been shown that uptake into the DNA is quantitatively related to the inhibition of the formation of infectious virions (28,29,30,31). Similarly the reluctance to adopt an alternating conformation may relate in part to the inability of some restriction enzymes to cleave sequences containing m\(6^A\). The recent crystal structure of the EcoRI-DNA complex has shown that before recognition of the base sequence, EcoRI kinks and unwinds the DNA adjacent to the sites of potential methylation (32). Thus methylation to form m\(6^A\) may play a dual role in protecting the DNA from restriction. It may alter the conformation to one which kinks and unwinds less readily, as well as preventing the formation of key hydrogen bonds (32).
Acknowledgements

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References