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^{1}H NMR and Circular Dichroism Studies of the B and Z Conformations of the Self-Complementary Deoxyhexanucleotide d(m^{5}C-G-C-G-m^{5}C-G): Mechanism of the Z–B–Coil Transitions†

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ABSTRACT: The double-helical conformations of d(m^{5}C-G-C-G-m^{5}C-G) in aqueous solution were studied by circular dichroism and ^{1}H NMR spectroscopy. In 0.1 M NaCl, only the B form is detected whereas the Z form is strongly predominant in 3 M NaCl. In the presence of 2 M NaCl, two resonance signals corresponding to the B and Z duplexes were observed for each proton below 50 °C, indicating a slow exchange between B and Z. However, the B–Z exchange becomes intermediate or fast in the 55–80 °C temperature interval. By contrast the exchange between B helix and single-stranded (or coil) forms is much faster for the same temperature conditions. The Z form is only detectable when the coil form is practically absent. With decreasing temperature the B form decreases in favor of the Z form. From proton line-width measurements under various experimental conditions, it was also shown that Z exchanges only with B, while the latter also exchanges with the single-stranded form (S): $Z \rightleftarrows B \rightleftarrows S$. The enthalpy value is about 8 ± 1 kcal/mol for the B–Z transition and about 40 ± 2 kcal/mol for the B–S dissociation (2 M NaCl solution). The activation energy is about 47 ± 2 kcal/mol for the $Z \rightarrow B$ and 39 ± 2 kcal/mol for the $B \rightarrow Z$ reaction. Very good agreement between the experimental results and computed data (based on the above kinetic reaction model) was found for the B, Z, and coil proportions. The B–Z transition of methylated $d(C\text{-}G)_n$ oligomers is only possible when the Watson–Crick hydrogen bonds between the CG base pairs are firmly maintained; otherwise, the transformation from B to Z would not occur, and B–S dissociation would take place instead.

In human DNA, 4–5% of cytosine residues are methylated (Tolstoshev et al., 1981), and methylated cytosines are frequently found preceding a guanine residue. It now appears that DNA methylation plays an important part in gene expression during development. For example, DNA in the region of duplicated γ-globin genes ($^G\gamma$ and $^A\gamma$) is relatively undermethylated in cells where these genes are expressed, whereas methylated DNA is found in adult bone marrow cells where these genes are inactive (Van der Ploeg et al., 1980). Ley et al. (1982) have recently shown that administration of 5-azacytidine to a patient with β-thalassemia reduces the frequency of methylation of specific cytosine residues and causes a striking increase in the synthesis of both $^G\gamma$-globin and $^A\gamma$-globin. These authors suggested that hypomethylation of DNA may be necessary for high level gene expression.

On the other hand Behe & Felsenfeld (1981) have shown that methylation on the 5-position of the cytosine residue in poly[d(G-C)] has the effect of inducing the transition from right-handed B-DNA into left-handed Z-DNA under physiological salt and pH conditions. In B-DNA duplexes, all residues adopt the S(C_2-endo) conformation for the sugar ring and the anti orientation for the bases. Conversely, in Z-DNA duplexes the deoxyguanosines take the N(C_3-endo) and syn conformations (Wang et al., 1979).

About 10 years ago, direct evidence of syn–anti equilibria of guanosine and adenine monophosphates in solution was obtained by the use of NOE[1] techniques (Tran-Dinh et al., 1972a; Guéron et al., 1973); the population of the anti conformation is favored in 5′-nucleotides. The correlation between syn/anti and N(C_3-endo)/S(C_2-endo) has also been studied on GMPs: the syn and N(C_3-endo) proportions are predom-

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[1] Abbreviations: d(m^{5}C-G-C-G-m^{5}C-G), 2′-deoxy-5-methylcytidyl(3′–5′)deoxyguanylyl(3′–5′)deoxycytidyl(3′–5′)deoxyguanylyl(3′–5′)-deoxy-5-methylcytidyl(3′–5′)deoxyguanine; NOE, nuclear Overhauser effect; ORD, optical rotatory dispersion; CD, circular dichroism; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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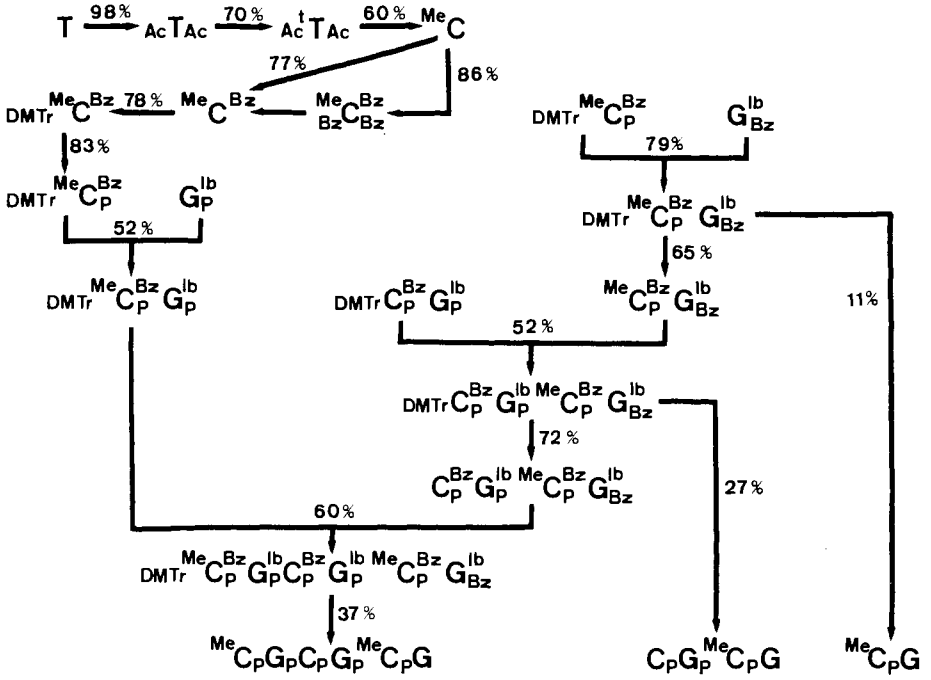
Scheme I



inant at acidic pH due to protonation of the base at N_7 while the anti S(C_2-endo) conformations are favored at neutral pH (Tran-Dinh et al., 1972b, 1975). During the same period, the conformation transition between R and L (two different double-helical forms) of poly[d(G-C)] was observed as a function of sodium salt concentration by Pohl & Jovin (1972) using ORD, CD, and UV techniques. The L form was later attributed to the Z form observed in X-ray diffraction analysis of $d(C-G)_3$ (Wang et al., 1979, 1981) and $d(C-G)_2$ (Drew et al., 1980; Crawford et al., 1980). Recently Fujii et al. (1982) also observed the Z form with a methylated hexamer, $d(C-G)_3$, in the solid state.

Following the pioneering work of Pohl and Jovin in 1972, several authors investigated the B–Z transition induced by chemical modification of bases such as 5-methylcytosine (Behe & Felsenfeld, 1981), 7-methylguanine (Möller et al., 1981), or 8-bromoguanine (Lafer et al., 1981), by carcinogen fixation (Sage & Leng, 1980), or by the addition of alcohol (Pohl, 1976; Ivanov & Minyat, 1981; Brahms et al., 1982), etc. However, although frequent guesses were made concerning the mechanism of the B–Z transition, very little information has been available until now. Several questions are still open to discussion. For example, (1) does the B–Z transition require the separation (or local separation) of the duplex by breaking of the Watson–Crick hydrogen bonds, the guanine base then rotating about the glycosidic bond according to the scheme B ⇄ S(B) ⇄ S(Z) ⇄ Z [where S(B) and S(Z) are the coil forms of the B and Z duplexes, respectively]? (2) Does the B–Z transition take place in the double-helical forms where the Watson–Crick hydrogen bonds are maintained? Is the Z duplex formed directly from the single-strand or from the A or B duplexes as an intermediate stage, especially in high salt solutions where the Z form is strongly predominant? Furthermore, in all NMR work carried out to date, the B and Z forms have been observed separately on low and high salt solutions, respectively. This means that the chemical shift comparison study between these two forms or between experimental results and computed data (Mitra et al., 1980, 1981; Sarma et al., 1981) is difficult since the salt effect on the local structure of molecules or on proton chemical shifts is still unknown.

In the present paper, in the hope of obtaining some information about the B–Z transition and facilitating the ^1H NMR assignment, the hexamer d(m^5C-G-C-G-m^5C-G) methylated at the 5-position of two external dC residues was synthetized, and the B and Z forms of this deoxyoligomer were subsequently studied in aqueous solution at various salt concentrations and temperatures, by CD and ^1H NMR techniques. With this hexamer we show that the B and Z forms can be observed simultaneously in solution by ^1H NMR or CD techniques and that the mechanism governing the B–Z transition, as well as the corresponding thermodynamic and kinetic parameters, can be deduced from the temperature effects on proton chemical shift, line width, B/Z ratio, etc.

Materials and Methods

The hexamer d(m^5CpGpCpGpm^5CpG) was synthesized in solution from dimers by using standard procedures with (triisopropylbenzenesulfonyl)nitrotriazole (TPSNT) as the coupling agent, according to Scheme I. 5-Methylcytidine was prepared from thymidine (Sung, 1981).

The deprotected hexamer was purified by G-10 gel filtration and preparative HPLC (Zorbax ODS 9.3 mm), exchanged to the ammonium form on a Dowex 50W NH_4^+, and lyophilized. The final purity, checked on an HS-5 18 analytical column is better than 99%. d(m^5C-G-C-G-m^5C-G) was dissolved in ^2H_2O containing 0.1 M or 2 M NaCl plus 5 mM PO_4^{2-} and was free of possible divalent ions by adding EDTA (≈0.1 mM). The pH was adjusted to 7–8 by the addition of a small amount of NaOH. The samples were lyophilized twice in ^2H_2O and redissolved in ^2H_2O at final concentration of 1.4–1.8 mM. These solutions were put into NMR tubes which were then degassed (on a vacuum line) and sealed. Proton chemical shifts and coupling constants were obtained from 500-MHz ^1H NMR spectra recorded on a Bruker WM500. The chemical shifts were measured at various temperatures from 3-(trimethylsilyl)[$^2H_4$]propionic acid (TMP). Proton longitudinal relaxation time experiments were performed by using the inversion recovery method (180°–τ–90°).

Circular dichroism was measured with an Autodichrograph Mark V (Jobin Yvon) spectropolarimeter. Samples for CD spectroscopy were prepared by diluting the oligomer in a

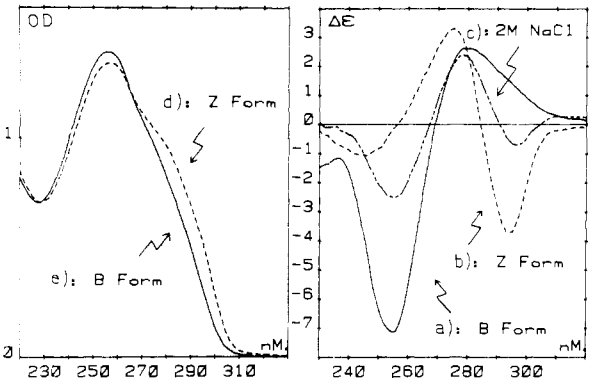


FIGURE 1: CD spectra of d(m^{5}C-G-C-G-m^{5}C-G) at 25 °C with (a) 0.1 M NaCl, (b) 3 M NaCl, and (c) 2 M NaCl. UV absorption spectra of the same hexamer at 25 °C: (d) Z form in 3 M NaCl and (e) B form in 0.1 M NaCl.

phosphate buffer containing 3 M NaCl. The CD spectra were recorded after heating the solution to 60 °C for 10 mn and then cooling it to room temperature for measurement. The UV absorption spectra were obtained on a Cary 219 spectrophotometer under the same experimental conditions as for the CD spectra.

Results

CD and UV Studies. The CD spectra of d(m^{5}C-G-C-G-m^{5}C-G) in 0.1, 2, and 3 M NaCl recorded at room temperature are presented in Figure 1a–c. The spectrum obtained at 0.1 M NaCl (Figure 1a) shows an important negative band at 255 nm and a positive band around 280 nm characteristic of a B form. In contrast at 3 M NaCl, a conservative spectrum is observed, with a positive band at 275 nm and a negative one at 296 nm (Figure 1b). This spectrum is similar to those obtained for poly[d(G-C)]·poly[d(G-C)] (Pohl & Jovin, 1972), and poly[d(m^{5}C-G)]·poly[d(m^{5}C-G)] (Behe & Felsenfeld, 1981) at high ionic strength. The CD spectrum obtained at 2 M NaCl (Figure 1c) is intermediate between those recorded at 0.1 and 3 M NaCl and shows that the B and Z proportions are almost equivalent. d(m^{5}C-G-C-G-m^{5}C-G) presents at 2 M NaCl a proportion of Z conformation that is obtained with the d(C-G)$_3$ only in higher salt concentration (3 M NaCl) (Quadrifoglio et al., 1981). This result is in good agreement with the shift to lower salt concentrations of the B–Z transition midpoint on methylation of poly[d(G-C)]·poly[d(G-C)] (Behe & Felsenfeld, 1981).

The UV absorption spectra of the oligomer at room temperature are presented in Figure 1d,e with 3 and 0.1 M NaCl solutions, respectively. The B–Z transition can be followed by the appearance of a characteristic shoulder at 290 nm as in the case of poly[d(C-G)] and methylated poly[d(C-G)].

Nonexchangeable ^{1}H NMR Studies. (1) Proton Assignments. (a) In 0.1 M NaCl Solution. Figure 2 shows the 500-MHz ^{1}H NMR spectrum of d(m^{5}C-G-C-G-m^{5}C-G) in the 0.1 M NaCl solution at 70 °C. The spectra obtained at low temperature (27 °C) are presented in Figures 4A and 5A and will be discussed later. Apart from the comparison with proton spectra of d(m^{5}C-G) and d(C-G-m^{5}C-G) (not shown) at high temperature (t > 70 °C) the proton assignment was performed by the methods described in our previous papers (Tran-Dinh et al., 1982a–c). In the first stage, proton signals of the same residue were determined from proton–proton decoupling, applied systematically to all proton resonances at three temperatures, 70, 27, and 2 °C. The identification of the initial and terminal residue signals is obtained straightforwardly by observing the exocyclic proton resonances or the

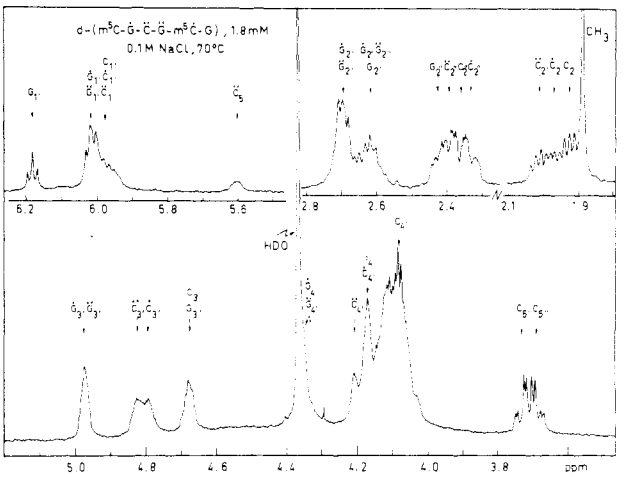


FIGURE 2: 500-MHz ^{1}H NMR spectrum of d(m^{5}C-G-C-G-m^{5}C-G) in neutral aqueous solution (1.8 mM, 0.1 M NaCl + 5 mM PO_4^{2-}, 70 °C). $G_{1'}$ = $H_{1'}$ of terminal dG, $\dot{G}_{1'}$ = $H_{1'}$ of internal dG, and $\ddot{G}_{1'}$ = $H_{1'}$ of central dG, etc.

$H_{3'}$ multiplet. The $H_{2'}$ and $H_{2''}$ resonances of the purine residues exhibit chemical shifts of 2.5–2.9 ppm while those of pyrimidine residues are located at higher fields, between 1.9 and 2.5 ppm. Except for the terminal residue, the $H_{3'}$ resonances of dG (or dA) are located at fields lower than those of dC (or dT). The same is true for the $H_{4'}$ resonances.

Apart from the main criteria mentioned above, comparison of proton chemical shifts relative to several DNA fragments studied in our laboratory [d(A-C-A-T-G-T) (Tran-Dinh et al., 1982b), d(C-C-A-T-G-G) (Tran-Dinh et al., 1982c), d(C-A-C-G-T-G) and d(G-T-G-C-A-C) (Tran-Dinh et al., 1983), and d(A-T-G-T) (Neumann et al., 1982)] allows us to formulate other criteria that will facilitate proton assignments for longer DNA fragments. For the present hexamer d(m^{5}C-G-C-G-m^{5}C-G) in particular, (i) the $H_{1'}$ resonance of the terminal residue is situated at a lower field than that of the same residue but is located in the internal or initial position, $\delta H_{1'}$ (pX) > $\delta H_{1'}$ (pXp,Xp) where X = dG, dA, dT, or dC, and (ii) the $H_{2''}$ resonance of the terminal *purine* residue lies in a higher field region with respect to the other $H_{2'}$ and $H_{2''}$ protons of the same purine residue in the initial or internal position, $\delta H_{2''}$ (pX) < $\delta H_{2''}$ (pXp,Xp) where X = dG or dA.

The base proton resonance assignments were determined by comparison of d(m^{5}C-G) with d(C-G-m^{5}C-G) and by longitudinal relaxation time and NOE measurements. The H_5 and H_6 resonances of central dC are characterized by a coupling constant of 7–8 Hz, absent on the H_6 resonance of the initial and internal m^{5}dC. The large chemical shift difference between two methyl groups or two H_6 protons of initial and internal m^{5}dC (0.2–0.3 ppm) allowed these proton resonances to be assigned by comparison with d(C-G-m^{5}C-G) proton spectra. The distinction between two H_6 and two CH_3 resonances of initial and internal m^{5}dC was confirmed by T_1 measurements at high temperature (t = 70 °C) where $\omega\tau_c$ < 1. T_1 values of 1.8 and 1.2 s were found for two H_6 of initial and internal m^{5}dC, respectively. Similarly, the G_8 resonance of terminal dG is characterized by a longer T_1 relaxation time (2.5 s) compared with that of internal (1.9 s) and central (1.7 s) dG residues (at t = 70 °C). The G_8 assignment was also verified on the basis of NOE experiments by saturating the $H_{2'}$ resonance of the same residue.

(b) In 2 M NaCl Solution. d(m^{5}C-G-C-G-m^{5}C-G) was dissolved in ^2H_2O containing 2 M NaCl plus 5 mM PO_4^{2-} at pH 7.1. The solution was heated to 95 °C and cooled slowly to a given temperature. A 30-min equilibration time was

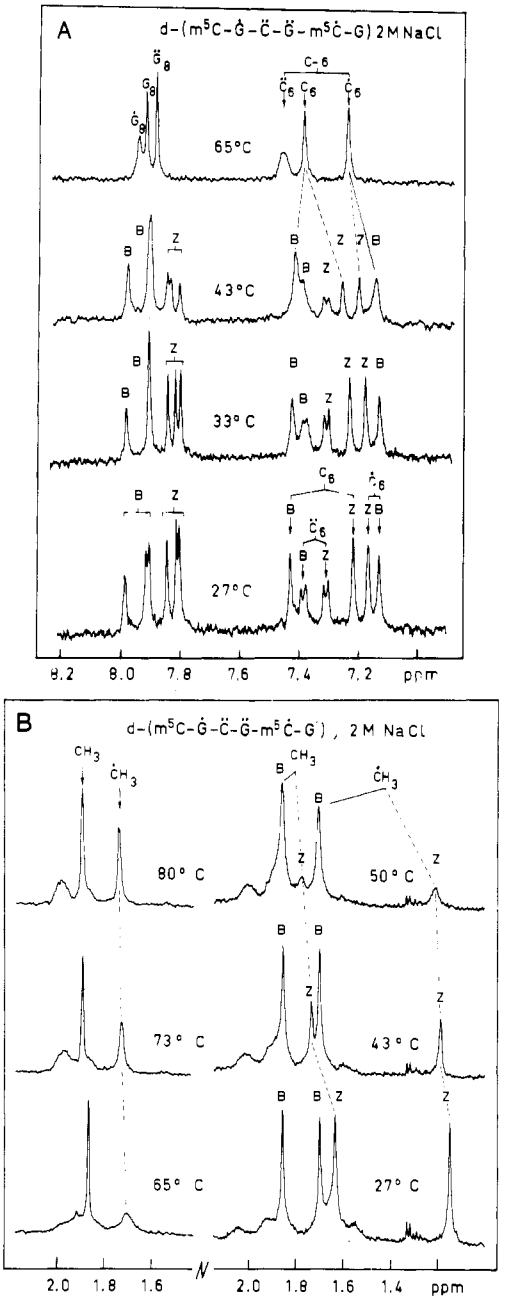


FIGURE 3: Temperature effect on 500-MHz ^1H NMR spectra of d(m^5C-G-C-G-m^5C-G) base protons in 2 M NaCl solution. (A) G_8 and C_6 proton spectra. $C_6 = H_6$ of initial dC, $\dot{C}_6 = H_6$ of internal dC, and $\ddot{C}_6 = H_6$ of central dC, etc. (B) Methyl proton spectra. CH_3 = methyl protons of initial dC, and $\dot{C}H_3$ = methyl protons of internal dC.

allowed after each temperature variation of 5 °C. Proton spectra were then recorded after verification of the absence of any change in chemical shift or a relative intensity between resonance signals. In the presence of 2 M NaCl or for $t >$ 60 °C, each proton gives rise to a single resonance signal with a chemical shift similar to that obtained with a 0.1 M NaCl solution. For $t <$ 50 °C, on the other hand, two resonance signals were observed for each base proton (Figure 3A,B). Figures 4 and 5 also show the $H_{1'}$, H_5, $H_{2'}$, and $H_{2''}$ spectra obtained with 0.1 (Figures 4A and 5A) and 2 M NaCl (Figures 4B and 5B) solutions.

The base and $H_{1'}$ proton resonances of the B and Z forms can be easily distinguished by (i) comparison with 0.1 M NaCl proton spectra where only the B form exists and (ii) the different variations in resonance intensity when the temperature is changed. The base and $H_{1'}$ resonance assignments of the

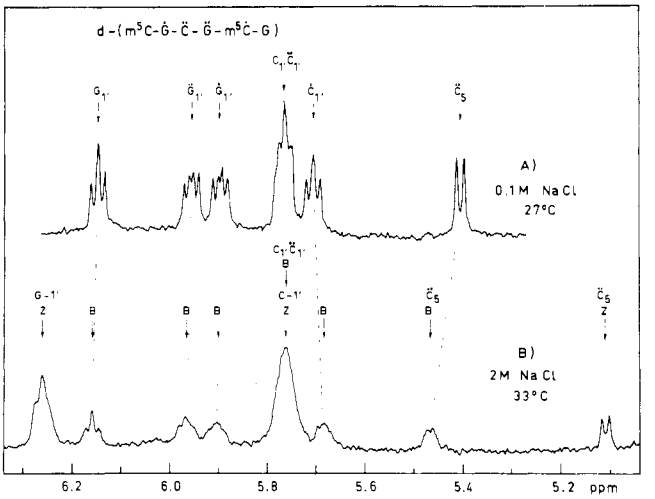


FIGURE 4: 500-MHz $H_{1'}$ and H_5 proton spectra of d(m^5C-G-C-G-m^5C-G). (A) In 0.1 M NaCl solution and (B) in 2 M NaCl solution.

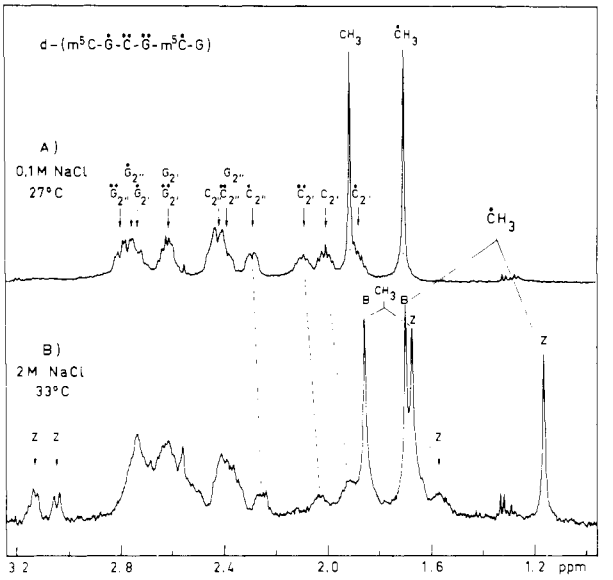


FIGURE 5: 500-MHz $H_{2'}$, $H_{2''}$, and CH_3 proton spectra of d(m^5-C-G-C-G-m^5C-G) in neutral solution. (A) With 0.1 M NaCl and (B) with 2 M NaCl. $G_{2'} = H_{2'}$ of terminal dG, $\dot{G}_{2'} = H_{2'}$ of internal dG, and $\ddot{G}_{2'} = H_{2'}$ of central dG, etc.

B form in 2 M NaCl are straightforward since the proton chemical shifts corresponding to the B form of d(m^5C-G-C-G-m^5C-G) are almost the same in both 0.1 M NaCl and 2 M NaCl solutions.

In the case of the Z form, the H_5 and H_6 resonances of the central dC residue are easily assigned owing to the J_{5-6} coupling constant of 7–8 Hz, which is of course absent on the H_6 resonance of the two m^5dC's. However, it is difficult to distinguish the two CH_3 and the two H_6 resonances of initial and internal m^5dC since shorter oligomers do not always give rise to the Z form under the same experimental conditions. The best way to assign the resonances of the Z form is to find corresponding signals from the B form by choosing a temperature interval in which a fast or intermediate exchange between B and Z takes place. Since the chemical shift difference between the B and Z forms a given proton i, $\Delta\delta i$ (B–Z), is not the same from one residue to another, the temperature effect on the line width should strongly depend on this parameter, $\Delta\delta i$ (B–Z): the larger $\Delta\delta i$ (B–Z) the greater the i proton line width. Figure 3B shows that as the temperature decreases from 80 to 65 °C, the CH_3 line width of initial m^5dC remains practically unchanged, while the CH_3

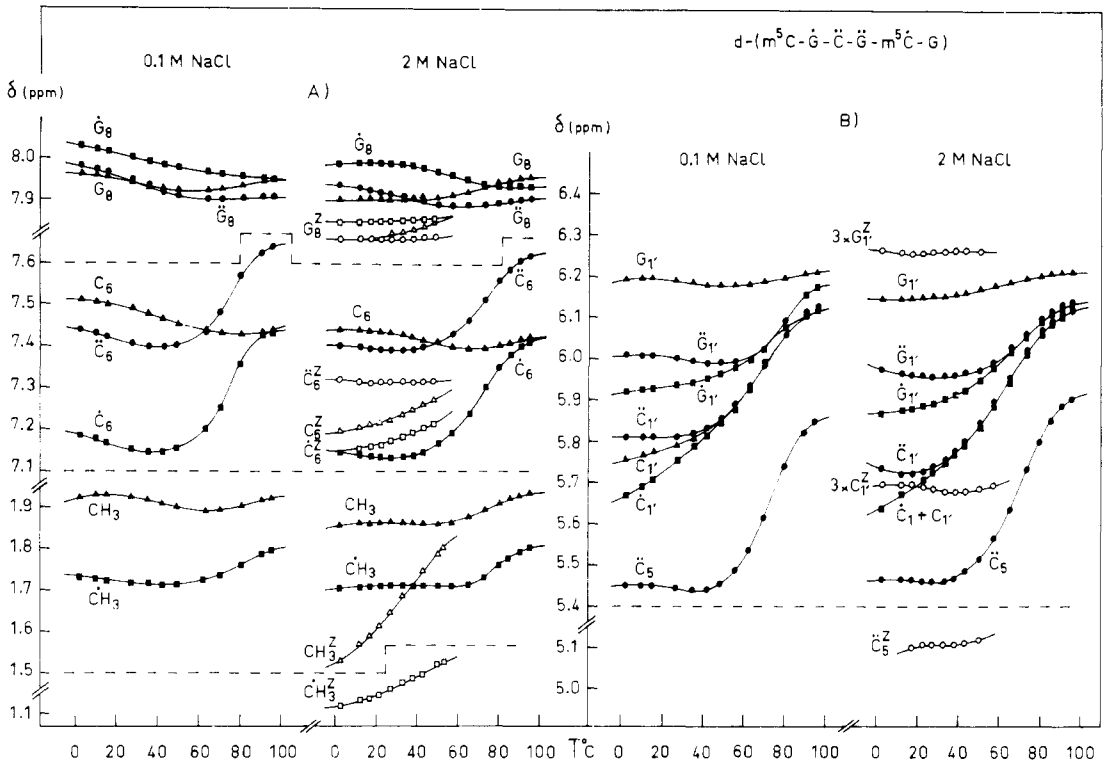


FIGURE 6: Temperature dependence of the proton chemical shifts of d(m^{5}C-G-C-G-m^{5}C-G) in 0.1 M NaCl and 2 M NaCl solutions. (A) Base protons and (B) $H_{1'}$ and H_5 protons. Protons are indicated as in Figures 3–5.

resonance of internal m^{5}dC broadens considerably. There are two main reasons that preclude that this line broadening is due to the exchange between the coil and the B double helix: (i) the chemical shift difference between the B double-helical and coil forms (0.1 ppm) is the same for both CH_3 groups with 2 M as well as with 0.1 M NaCl; (ii) in 0.1 M NaCl solution the line broadening is insignificant and is the same for both CH_3 groups. In fact, the substantial line broadening of the internal m^{5}dC CH_3 resonance should be due to the exchange between B and Z, and the CH_3 resonance of the internal m^{5}dC residue in the B form should have a corresponding signal in the Z form located far away at 1.14 ppm. The other CH_3 (Z) signal situated close to those of the B form was thus assigned to the initial residue. Once the distinction between the two CH_3 groups was made, the H_6 assignment for two m^{5}dC residues, by NOE or decoupling experiments (with saturation of CH_3 resonances), became straightforward.

Figure 4B shows that the $H_{1'}$ resonances of three dG's and three dC's, respectively, overlap in the Z form. On the basis of the ring current effect of the guanine, the resonances situated at lower field, 6.26 ppm, were attributed to the dG $H_{1'}$ and the others, at 5.78 ppm, to the dC $H_{1'}$. This assignment is confirmed by NOE experiments by saturation of the $G_{1'}$ (Z) resonances (see below). We are unable to assign individual $H_{2'}$ and $H_{2''}$ resonances corresponding to the Z form at this time since below 50 °C, where the Z form is detectable, the $H_{2'}$ and $H_{2''}$ multiplets are poorly resolved and overlap with those of the B form.

(*2*) *Temperature Effects.* (*a*) *On Proton Chemical Shifts.* The nonexchangeable proton chemical shifts in 0.1 and 2 M NaCl solutions were measured as a function of temperature between 2 and 95 °C. In Figure 6 the chemical shifts of the base and $H_{1'}$ protons are plotted vs. temperature. The results show that the proton chemical shifts corresponding to the B form in 0.1 and 2 M NaCl solutions are practically the same at high ($t \geq 90$ °C) or at low ($t < 40$ °C) temperature where only one species (duplex or single strand) is present. For intermediate temperatures (40 °C $< t <$ 90 °C), the slope of the sigmoidal curves and the midpoint temperature for the B double helix–coil transition are slightly different. The $t_{1/2}$ value is about 73 °C for 0.1 M and 70 °C for 2 M NaCl solutions.

Except for the CH_3 resonance of initial m^{5}dC, the proton resonances of the Z form display only a small shift variation; in particular, the proton chemical shift corresponding to the central residues (G_8, C_6, C_5, $G_{1'}$, $C_{1'}$) are virtually independent of temperature between 2 and 50 °C, where the Z form is detectable. These results strongly suggest that the Z double helix–coil transition does not take place in this temperature range.

(*b*) *On Proton Line Widths and B/Z Proportions.* As the temperature decreases from 80 to 20 °C, several protons, particularly H_6 and CH_3 of initial and internal m^{5}dC and H_5 of central dC, exhibit large line-width variations as indicated in Figure 7 for the Z and B forms in 2 and 0.1 M NaCl solutions. In the case of the B form, the line broadening is different from one proton to another and is clearly larger with 2 M NaCl than with 0.1 M NaCl. By contrast, the line-width variation is practically the same for all proton resonances of the Z form. It should be emphasized here that in the case of the 0.1 M NaCl solution at 25 °C where only the B double-helix form is present, the proton line widths are only 2–3 Hz. This is similar to the case of the other self-complementary hexamers such as d(A-C-A-T-G-T) (Tran-Dinh et al., 1982b), d(C-C-A-T-G-G) (Tran-Dinh et al., 1982c), and d(C-A-C-G-T-G) and d(G-T-G-C-A-C) (Tran-Dinh et al., 1983). By contrast, the long double helices of poly[d(G-C)] exhibit broad proton resonance signals whose line widths are at least 10 Hz at 45 °C (Patel et al., 1982a,b). These results suggest that an intermolecular association forming a long double helix in which one single strand of d(m^{5}C-G-C-G-m^{5}C-G) binds with two other strands (and so on) does not occur to a significant extent.

Below 50 °C where the coil proportion α is negligible, the proportions of B and Z can be determined by integrating the

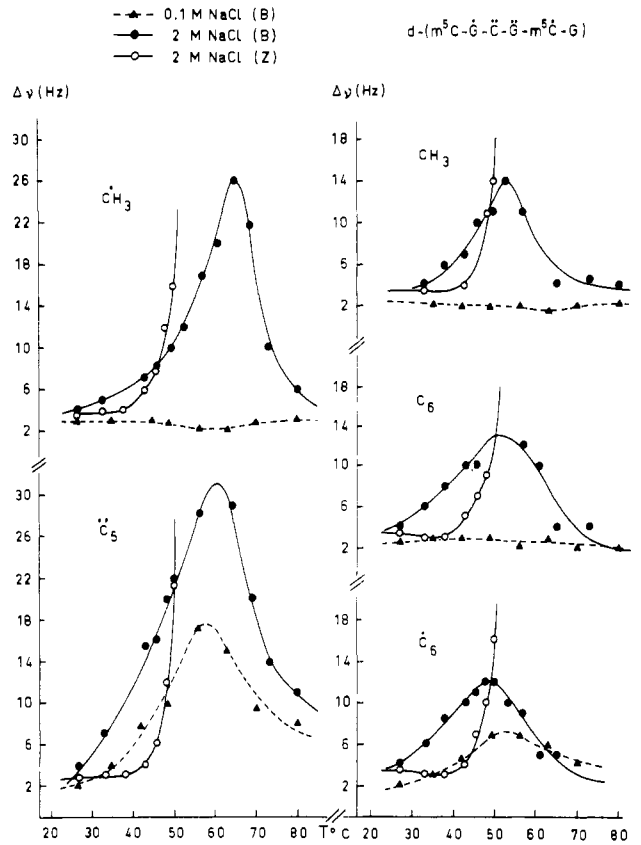


FIGURE 7: Temperature effect on proton line widths of the B and Z forms of d(m^{5}C-G-C-G-m^{5}C-G) in 0.1 and 2 M NaCl solutions.

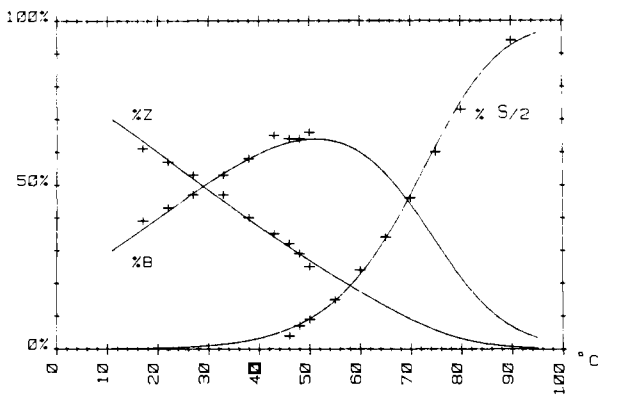


FIGURE 8: Experimental (+) and computed (–) values of the B, Z, and coil proportions of d(m^{5}C-G-C-G-m^{5}C-G) in 2 M NaCl solution. The coil proportion, S, is divided by a factor of 2; thus, the total proportion is B + Z + S/2 = 100% (see text).

signals of H_8 (dG), H_6 (dC or m^{5}dC), and possibly CH_3 (m^{5}dC). The proportions of the Z, B, and α in 2 M NaCl solution are plotted vs. temperature in Figure 8. We find that lowering the temperature has the effect of increasing the proportion of Z to the detriment of B. The Z proportion is only about 25% at 50 °C and increases to 61% at 17 °C.

Discussion

B–Z Conformations. In the 2–27 °C temperature range, where only the B double-helical form is present in 0.1 M NaCl solution, the $H_{1'}$ resonances of three dG residues are well separated. This allows the sugar conformation to be determined from proton–proton coupling constants. The $J_{1'2'}$ value of about 9.0 ± 0.2 Hz for the internal and central dG corresponds to a proportion of S($C_{2'}$-endo) of about 85%, a value slightly higher than those observed in other hexamers such as d(A-C-A-T-G-T), d(C-C-A-T-G-G), d(C-A-C-G-T-G), and

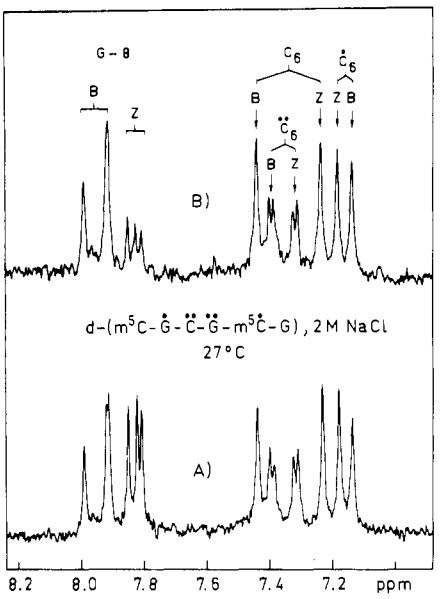


FIGURE 9: Negative NOE enhancement on the G_8 (Z) proton signals upon saturation of the $G_{1'}$ (Z) resonances of d(m^5-C-G-C-G-m^{5}C-G) in 2 M NaCl solution at 27 °C. (A) Without saturation and (B) with saturation of the $G_{1'}$ (Z) resonances at 6.26 ppm.

d-(G-T-G-C-A-C) (Tran-Dinh et al., 1982b,c, 1983). The $J_{1'2'}$ value of the terminal dG is only about 7.2 ± 0.2 Hz, clearly smaller than those of the other dG residues and corresponding to an S proportion of about 70%. In addition these $C_{1'}$ resonances are well separated below 20 °C (not shown). Although the resolution is relatively poor in this temperature range, the sum of $J_{1'2'} + J_{1'2''}$ can be estimated as approximately 14 Hz. These results show that the S conformation ($C_{2'}$-endo) is largely predominant for all residues and the double helices of d(m^{5}C-G-C-G-m^{5}C-G) adopt the B form in 0.1 M NaCl solution. The fact that proton chemical shifts and $J_{1'2'} + J_{1'2''}$ coupling constants are very similar between 0.1 M and 2 M NaCl (Figures 4A and 4B) indicates that the B double-helical conformation remains unchanged in this salt concentration range.

In the case of the Z form the overlapping of three $G_{1'}$ resonances prevents the proportion of N($C_{3'}$-endo) conformation from being evaluated. However, on saturation of the $G_{1'}$ resonances at 6.26 ppm, a negative NOE of 53 ± 2% was observed on the G_8 resonances (Figure 9). This result confirms the $G_{1'}$ and $C_{1'}$ resonance assignment based on the ring current effect and at the same time demonstrates that the guanine bases in the Z form adopt the syn conformation in solution as well as in the solid state. Similar results have also been obtained in studies of poly[d(G-C)] and poly[d(G-m^{5}C)] by ^{1}H NMR (Patel et al., 1982a).

Proton Chemical Shift Comparison between the B and Z Double Helices. The simultaneous observation of the B and Z forms of d(m^{5}C-G-C-G-m^{5}C-G) in 2 M NaCl solution provides an opportunity for a chemical shift comparison study between B and Z as well as between experimental results and computed data. It follows from Figure 3A that the chemical shift difference between the syn and anti conformation is surprisingly not very large for the G_8 resonances: the signals corresponding to the Z form are situated about 0.10–0.15 ppm upfield from those of the B form. In the case of the H_6 resonances their relative positions for the B and Z conformations depend on the position of the dC residue in the sequence: the C_6 (Z) resonance is situated at lower field than the C_6 (B) signal for internal m^{5}dC, whereas the C_6 (Z) resonances of initial m^{5}dC and central dC are situated upfield

Table I: Proton Chemical Shift Differences (in ppm) between the B and Z Forms [δ(B) − δ(Z)] of d(m⁵C-G-C-G-m⁵C-G), d(G-C)₈, and Poly[d(G-C)]

proton	d(m⁵C-G-C-G-m⁵C-G) (central residue)	d(G-C)₈ [a]	poly[d(G-C)] [b] without x correction	poly[d(G-C)] [b] with x correction
G_8	+0.10	≈0	−0.25	0.41
C_5	+0.35	+0.14	+0.14	0.15
C_6	+0.08	≈0	+0.51	+0.51
$G_{1'}$	−0.30	−0.39	−0.39	0.06
$C_{1'}$	+0.05	≈0	+0.21	0.21

[a] Patel et al. (1982a) (experimental values). [b] Mitra et al. (1981) (computed values).

from those of the B form. The fact that the methyl group of internal m⁵dC is located at highest field (δ = 1.14) very far from the B form signal (δ = 1.71) suggests that this methyl group is very close to (but situated "inside") the guanine ring (upfield shift) of the neighboring dG residue. This hypothesis is supported by a recent X-ray study in which Fujii et al. (1982) showed that the methyl groups in d(m⁵C-G)₃ are tucked under and in close van der Waals contact with the imidazole ring of guanine. Similarly, by the same chemical shift consideration the H_5 proton of central dC should be located in the proximity of the dG imidazole ring. However, the distance between the H_5 proton and the imidazole ring of the neighboring dG would be greater for the internal CH_3 group since the ring current effect is clearly stronger in the latter case: the chemical shift difference between the B and Z forms is only 0.35 ppm for the H_5 proton instead of 0.57 ppm for the internal m⁵dC methyl group. In addition, in contrast to the case of the B form where all the $H_{1'}$ resonances are well separated at low temperature, the close proximity of three G_8 and three C_6 signals and the supperposition of three $G_{1'}$ or three $C_{1'}$ signals in the Z form firmly indicate that the magnetic environment of a given proton in the same family of residue is practically identical and the structure is more ordered in the Z than in the B duplexes.

The chemical shifts of the central dG and dC protons (G_8, $G_{1'}$, C_5, C_6, $C_{1'}$) of d(m⁵C-G-C-G-m⁵C-G) in the Z form were found to be practically identical with those obtained from d(G-C)₈ in 4 M NaCl under the same temperature conditions (Patel et al., 1979). These results thus confirm the Z structure of the d(G-C)₈ oligomer at high salt concentrations, similar to the case of poly[d(G-C)] (Patel et al., 1982a,b). Recently Mitra et al. (1981) have computed, with and without correction for the change in the sugar–base torsion angle (χ), the theoretical chemical shift of the base and $H_{1'}$ protons for the B and Z conformations of poly[d(G-C)] and predicted the relative position of the B and Z proton resonances. Table I shows the experimental results for the chemical shift differences between B and Z, Δ[δ_i(B) − δ_i(Z)], for the central residue base and $H_{1'}$ protons of d(m⁵C-G-C-G-m⁵C-G), along with those of d(GC)₈ (Patel et al., 1979) and the theoretical data for poly[d(GC)] (Mitra et al., 1981). The results in Table I show that the chemical shift difference between the B and Z forms of d(m⁵C-G-C-G-m⁵C-G) and d(GC)₈ is practically the same for G_8, $G_{1'}$, C_6, and $C_{1'}$ and is slightly different for the C_5 proton. This slight difference may be due to the fact that in the case of d(GC)₈ the experimental conditions (salt concentrations, pH, etc.) were not the same for the B and Z observations.

As far as the theoretical data are concerned, without any base–sugar torsion angle correction, the agreement is satisfactory for $G_{1'}$ and C_5, mediocre for C_6, $C_{1'}$, and C_5, and poor

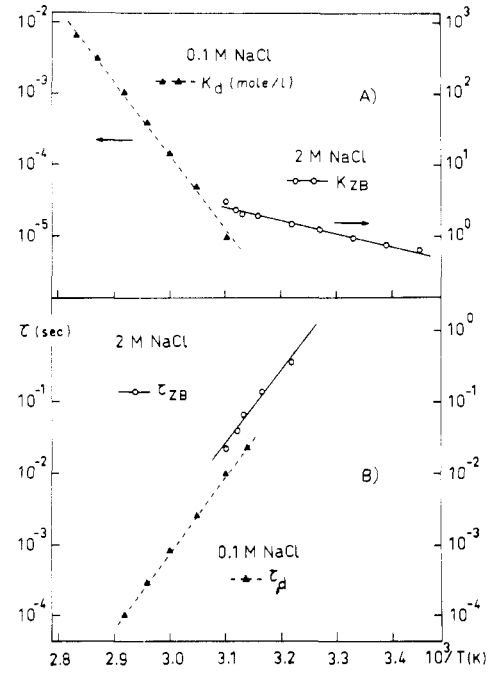


FIGURE 10: Thermodynamic at kinetic parameters corresponding to the B–coil and B–Z transitions of d(m⁵C-G-C-G-m⁵C-G). (A) Semilog plot of the B–coil dissociation constant, K_d (in 0.1 M NaCl), and the B/Z equilibrium constant, K_{ZB} (in 2 M NaCl), vs. the reciprocal absolute temperature. (B) Semilog plot of the lifetime τ_d of the B form in 0.1 M NaCl and the exchange time τ_{ZB} and τ_{BZ} in 2 M NaCl solutions vs. the reciprocal absolute temperature (see text).

for G_8. The relative position of the G_8 resonances of the B and Z configurations should be reversed. The correction for the torsion angle effect alters only the terms related to the dG residue. The relative position of the G_8 resonances for the B and Z forms is found to be correct. However, the difference in chemical shift of the G_8 resonances between B and Z is fairly large, while that of $G_{1'}$ becomes too small.

B Double Helix–Coil (B–S) and B–Z Transitions. (1) B–S Transition. In 0.1 M NaCl solution, the CD and ^{1}H NMR results show that only the B form exists at low temperature. The coil proportion α can be determined from the melting curves (Figure 6) according to the following relation (Tran-Dinh et al., 1982b,c):

$$\delta_{obsd} = \alpha\delta_c + (1 - \alpha)\delta_h \quad (1)$$

where δ_c and δ_h are the chemical shifts of the coil and helical forms measured at high ($t \approx 100$ °C) and at low ($t \approx 20$ °C) temperatures, respectively. Figure 10 shows the semilog plot of the dissociation constant K_d vs. temperature [$K_d = 2\alpha^2 c/(1 - \alpha)$, where c = concentration of d(m⁵C-G-C-G-m⁵C-G) = 1.8 mM]. A dissociation enthalpy of 48 kcal/mol was found for d(m⁵-C-G-C-G-m⁵C-G) in 0.1 M NaCl solution. The enthalpy value for 2 M NaCl solution is smaller, about 40 kcal/mol. The fact that the B helix–coil dissolution enthalpy corresponds well to the maximum value of 7 or 8 kcal/mol for each base pair and that the B helix–coil midpoint transition temperatures, $t_{1/2}$, in 0.1 M NaCl and in 2 M NaCl solutions are much higher than those of the other self-complementary hexamers (Tran-Dinh et al., 1982b,c, 1983) (Patel, 1975) clearly indicates that d(m⁵C-G-C-G-m⁵C-G) helices contain six base pairs. An intermolecular association process to form a very long chain in which a single strand binds with two others (four residues bind with the first strand while the two other residues with the second strand and so on) would correspond to a much smaller helix–coil dissociation enthalpy and a much lower midpoint transition temperature and is therefore negligible for the present hexamer. This conclusion is also sup-

ported by proton line-width measurements mentioned in Figure 8.

From the variation in line width of the central dC H_5 proton, the lifetime τ_d of the B double-helical form (in 0.1 M NaCl solution) can be deduced from the following relationship (Tran-Dinh et al., 1983):

$$\frac{1}{T_{2,obsd}} = \frac{\alpha}{T_{2s}} + \frac{1-\alpha}{T_{2d}} + \alpha^2(1-\alpha^2)4\pi^2\Delta\nu^2\tau_d \qquad (2)$$

where T_{2s} and T_{2d} are the spin–spin relaxation times of the coil and helix forms, respectively, α is the coil proportion, and $\Delta\nu$ is the chemical shift difference (in Hz) between the coil and helical forms. $T_{2,obsd}$ is determined from the effective observed proton line width, W, of the internal m^5dC H_6 and central dC H_5 resonances ($1/T_{2,obsd} = \pi W$). In the latter case W is equal to the difference between the proton line width at half-height and the J_{5-6} coupling constant (about 7.5 Hz). T_{2d} and T_{2s} can be obtained from the proton line widths at 25 and 95 °C, respectively. For intermediate temperatures, approximate values for T_{2d} and T_{2s} can be calculated by assuming the coil and helix motion to be isotropic with an activation energy of about 4 (coil form) and 7 kcal/mol (helix form) (Neumann & Tran-Dinh, 1981, 1982). However, we found that this correction does not have a significant effect since the error on the line-width measurements is generally greater than the variation of the T_{2d} and T_{2s} values in the 25–95 °C temperature range. In Figure 10B the τ_d values are plotted vs. the reciprocal absolute temperature. The slope of this curve corresponds to an activation energy of 49 ± 3 kcal/mol for the B double helix–coil dissociation reaction. The τ_d value at the transition temperature is about 1×10^{-4} s, similar to the case of d(C-A-C-G-T-G) and d(G-T-G-C-A-C) (Tran-Dinh et al., 1983).

(2) B–Z Transition. The simultaneous observation of two distinct signals for the B and Z duplexes below 50 °C indicates that the exchange between B and Z is slow in this temperature range. By contrast the exchange between the double helix and coil forms is much faster since only one signal was observed for each proton in 0.1 M NaCl as well as in 2 M NaCl (for the "B" form signals) between 2 and 95 °C. The large line-width variation for the B proton resonances in 2 M NaCl can be explained by the fact that in 0.1 M NaCl solution the line widths depend on the exchange rate between the B double helix and the coil, whereas in 2 M NaCl solution, the exchange between B and Z also contributes to the line broadening. Above 55 °C the B–Z exchange can be intermediate or fast, and only one signal was observed for each proton. As a result the proton line width also depends on the chemical shift difference between the B and Z forms, as mentioned above for the methyl resonances (Figure 3b).

For the proton resonances of the Z form, the slow exchange between Z and B below 50 °C means that the observed line width is equal to the sum of the natural line width and the exchange frequency from Z to B:

$$\pi\Delta\nu_{obsd} = \frac{1}{T_{2,obsd}} = \frac{1}{T_{2(Z)}} + \frac{1}{\tau_{Z\to B}} \qquad (3)$$

where $1/T_{2(Z)} = \pi\Delta\nu$ is the natural proton line width corresponding to the Z form and $\tau_{Z\to B}$ is the exchange time from Z to B. Since the natural line width is only about 1–2 Hz, its contribution to the observed line width (10–25 Hz) is negligible in the 40–55 °C temperature interval. Hence, the line broadening of Z proton resonances mainly reflects the exchange frequency or the exchange time $\tau_{Z\to B}$ from Z to B, and the observed line width was found to be practically the same for all resonances of the Z form.

In brief, (i) the Z double-helical form is only detectable below 50 °C where the single-stranded form is practically absent, and no evidence for a Z double helix–coil transition was found on the basis of chemical shift consideration, (ii) as the temperature is lowered the B proportion decreases in favor of the Z form, and (iii) it is shown from proton line-width measurements that Z exchanges only with B while the latter also exchanges with the coil form. On the basis of the above arguments, the mechanism for the exchange between the Z, B, and coil forms can be described as follows:

$$Z \underset{k_{B\to Z}}{\overset{k_{Z\to B}}{\rightleftharpoons}} B \underset{k_s}{\overset{k_d}{\rightleftharpoons}} S \qquad (4)$$

where S is the coil or single-stranded form.

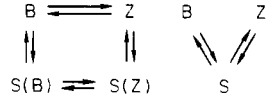
The equilibrium constant K_{ZB} can be determined; this is especially easy below 50 °C where the coil proportion is zero or negligible:

$$K_{ZB} = \frac{[B]}{[Z]} \qquad (5)$$

In Figure 10 the semilog plot of the ratio B/Z is given vs. the reciprocal absolute temperature. The linearity of this curve shows that the enthalpy of the Z ↔ B reaction is temperature independent and is about 8 ± 1 kcal/mol. At 29 °C (T_0 = 302 K) where the B/Z equilibrium constant K is equal to 1, the free energy change, $\Delta G_0 = -RT_0 \log K = \Delta H_0 - T_0\Delta S_0$, is zero for the Z–B reaction. This permits the entropy change, ΔS_0, of about 26.5 cal/mol to be deduced. It should be mentioned that in the case of poly[d(G-C)]·poly[d(G-C)], no significant temperature effect was found for the B–Z equilibrium between 20 and 40 °C (Pohl & Jovin, 1972). This difference between d(m^5C-G-C-G-m^5C-G) and poly[d(G-C)] may be due to the methyl group in methylated cytosine which stabilized the Z form in solution.

The exchange time τ_{ZB} can also be determined independently from proton line widths corresponding to the Z form. Figure 10 also shows the average τ_{ZB} values (deduced from the line widths of CH_3, H_6 of initial and internal m^5dC, and C_5 of central dC) vs. the reciprocal absolute temperature. As $\tau_{ZB}^{-1} = k_{Z\to B}$, where $k_{Z\to B}$ is the rate constant of the Z→B first-order reaction in the above kinetic model, the activation energy of this reaction was found to be about 47 kcal/mol.

On the basis of the above thermodynamic data, using (i) the enthalpy values of 8 and 40 kcal/mol for the B–Z transition and B–S (coil) dissociation, respectively (2 M NaCl solution) and the B–Z entropy value of 26.5 cal/mol and (ii) the experimental value of 70 °C for the B helix–coil midpoint transition temperature (with 2 M NaCl solution), we computed and reported the B, Z, and coil proportions vs. temperature in Figure 8. Very good agreement between the experimental and theoretical data is found for the B/Z ratio and coil proportion, α (Figure 8). We have tested other reaction models such as



where S(B) and S(Z) are the coil forms of the B and Z duplexes, respectively. However, no agreement between the experimental and theoretical data was obtained.

Conclusion

In the light of the above results and discussion we may conclude that the Z double helix is directly obtained from the B duplex without passing through the single-stranded form as an intermediate stage. Using the electropheresis technique,

Simpson & Shindo (1980) did not observe the hairpin formation during the B–Z transition of poly[d(G-C)] and came to a similar conclusion that the B–Z transition of poly[d(G-C)] is an intramolecular rearrangement, not requiring complete strand separation. Energetically speaking, if the B–Z transition process involves the local (or total) separation of the Watson–Crick hydrogen bonds of one or two base pairs, the Z form would be favored at high temperatures where the hydrogen bonds are less stable. On the basis of the above thermodynamic and kinetic data, it may be concluded that the B–Z transition is only possible when the Watson–Crick hydrogen bonds between the CG base pairs are firmly maintained; otherwise, the transformation from B to Z would fail, and the B helix–coil dissociation would take place instead. This conclusion is strongly supported by the fact that alternating $d(T\text{-}A)_n$ oligomers cannot give rise to the Z conformation since there are only two hydrogen bonds in an A·T base pair instead of the three present in G·C. By contrast a salt-induced B–Z transition occurs in the case of the self-complementary hexanucleotide consisting of thymidine and 2-aminodeoxyadenosine, $d(T\text{-}2\text{-}amino\text{-}A)_3$ (Gaffney et al., 1982).

In the case of short oligomers such as the present hexamer it is difficult to imagine that the B–Z transition is achieved by the flip of only one group of two base pairs while the other base pairs remain for a long time in the B configuration. This means that the B–Z flip is very fast and the whole helix was in either the B or the Z conformation. However, in the case of synthetic polymers such as poly[d(G-C)] and poly[d(m^5C-G)] the B and Z structures might be observed simultaneously in a long double helix. X-ray (Drew et al., 1981) and ^{1}H and ^{31}P NMR studies (Patel et al., 1982b) on d(C-G-C-G-A-A-T-T-C-G-C-G) showed that the double helix of this dodecamer adopts the B conformation although the CGCG and AATT segments were the potential candidates for Z and A duplexes, respectively. Similarly, for d(A-T-A-T-A-T-C-G-C-G-C-G), when only the double-helical form is present, this oligomer adopts the B form, whereas with increasing temperature the Z form was detected when the B form promoter, $d(AT)_3$, was already found in the single-stranded state (Quadrifoglio et al., 1981). It seems likely that the boundary zone between B and Z must spread over several residues, may be one helical turn, for the right- and left-handed forms to be simultaneously present in the same duplex.

Acknowledgments

We thank Dr. A. W. Rutherford for helping to correct the English in the manuscript.

Registry No. d(m^5C-G-C-G-m^5C-G), 88729-54-8.

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Sequential Resonance Assignments in ^{1}H NMR Spectra of Oligonucleotides by Two-Dimensional NMR Spectroscopy†

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ABSTRACT: A sequential assignment procedure is outlined, based on two-dimensional NOE (NOESY) and two-dimensional *J*-correlated spectroscopy (COSY), for assigning the nonexchangeable proton resonances in NMR spectra of oligonucleotides. As presented here the method is generally applicable to right-handed helical oligonucleotides of intermediate size. We applied it to a *lac* operator DNA fragment consisting of d(TGAGCGG) and d(CCGCTCA) and obtained complete assignments for the adenine H8, guanine H8, cytosine H6 and H5, thymine H6 and 5-methyl, and the deoxyribose H1′, H2′, H2″, H3′, and H4′ resonances, as well as some H5′, H5″ (pairwise) assignments. These assignments are required for the analysis of two-dimensional NOE and *J*-coupling data in terms of the solution structure of oligonucleotides.

Although potentially NMR gives the most detailed information on the structure of biomolecules in solution, the analysis of NMR spectra has been hampered by the difficulty of obtaining resonance assignments. For proteins this situation has changed recently as two-dimensional NMR techniques have made it possible to translate the known primary structure of the molecule into resonance assignments via so-called sequential-assignment methods (Wüthrich et al., 1982). The same techniques can be used to yield information about secondary and tertiary protein structure, as demonstrated recently for the *lac* repressor headpiece (Zuiderweg et al., 1983).

Feigon et al. (1982) were the first to use 2-D NMR[1] in the study of a double-stranded oligonucleotide and observed some interesting intra- and internucleotide NOE's. In this paper we wish to present a systematic method based on 2-D NMR for assigning resonances of the nonexchangeable protons in nucleic acids.[2] Thus far most of the attention of NMR spectroscopists has been directed to the low-field region of nucleic acid spectra, where the imino protons resonate that take part in the hydrogen bonds of Watson–Crick base pairs. Assignment strategies for these protons have been based upon chemical-shift calculations (Bell et al., 1983) or the study of melting behavior (Zuiderweg et al., 1981) and, more recently, on the nuclear Overhauser effect (NOE) (Redfield et al., 1981; Patel et al., 1982), which is observable between imino protons in neighboring base pairs. However, the cross-relaxation network formed by these protons does not extend to the exterior regions of the molecule, and of the nonexchangeable protons only the adenine H2 protons can be assigned via this approach.

The sequential assignment procedure presented here for the remaining, nonexchangeable protons in nucleic acids can be applied to right-handed helical oligonucleotides. Its application will be demonstrated for a seven base pair DNA duplex consisting of the two complementary heptanucleotides d(TGAGCGG) and d(CCGCTCA), where it yields the assignments of all purine H8, pyrimidine H6, cytosine H5, thymine CH_3, and deoxyribose H1′, H2′, H2″, H3′, and H4′ resonances, as well as some of the H5′ and H5″ resonances. These assignments may form the basis of a more detailed study of the structure of the DNA duplex in solution.

Materials and Methods

^{1}H NMR spectra were recorded at 360 MHz on a Bruker HX360 and at 500 MHz on a Bruker WM500 spectrometer, equipped with Aspect 2000 computers.

Two-dimensional NOE spectra were recorded by using the pulse sequence $(\pi/2\text{–}t_1\text{–}\pi/2\text{–}\tau_m\text{–}\pi/2\text{–Acq})_n$, described by Macura & Ernst (1980). Phase cycling was performed according to States et al. (1982); the carrier position was at the low-field side of the spectrum, so that only real t_1 data had to be collected. A total of 512 FID's, 2048 data points each, was recorded for each spectrum. These were transferred to a Cyber 170-760 computer via magnetic tape for processing. Before Fourier transformation the FID's were weighed with a 45–60°-shifted sine-bell function and zero filled to 4096 points. After phase correction the real matrix was transposed, and the interferograms were weighed with a similar sine bell, zero-filled to 2048 points, and Fourier transformed. Phase correction and symmetrization then yield a 1024 × 1024 spectrum in pure absorption phase.

COSY spectra were recorded with the $(\pi/2\text{–}t_1\text{–}\pi/2\text{–Acq})_n$ sequence described by Aue et al. (1976). A total of 512 FID's was recorded, each of 2048 points, with the carrier frequency outside the spectrum. Both FID's and interferograms were weighed with an unshifted sine-bell function before zero filling and Fourier transformation. Spectra are presented in the absolute-value mode and symmetrized.

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[1] Abbreviations: FID, free induction decay; 2-D, two-dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; COSY, two-dimensional *J*-correlated spectroscopy; ppm, parts per million; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; bp, base pair; A, adenine, T, thymine; C, cytosine; G, guanine; B, purine H8 (AH8 or GH8) or pyrimidine H6 (CH6 or TH6).

[2] For a preliminary report of this work, see Scheek et al. (1983).

0006-2960/84/0423-1371$01.50/0