Non-Watson–Crick Structures in Oligodeoxynucleotides: Self-Association of d(TpCpGpA) Stabilized at Acidic pH†

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ABSTRACT: The ^{1}H NMR spectrum of the tetradeoxynucleotide d(TpCpGpA) was examined as a function of temperature, pH, and concentration. At pH 7 and above the solution conformation for this oligodeoxynucleotide appears to be a mixture of random coil and Watson–Crick duplex. At 25 °C, a pH titration of d(TpCpGpA) shows that distinct conformational changes occur as the pH is lowered below 7.0. These conformational changes are reversible upon readjusting the pH to neutrality, indicating the presence of a pH-dependent set of conformational equilibria. At 25 °C, the various conformational states in the mixture are in rapid exchange on the NMR time scale. Examination of the titration curve shows the presence of distinct conformational states at pH greater than 7, and between pH 4 and pH 5. At pH <4, a third conformational state is present. When the pH titration is repeated at 5 °C, the conformational equilibria are in slow exchange on the NMR time scale; distinct signals from each conformational state are observable. The stable conformational state present between pH 4 and pH 5 represents an ordered conformation of d(TpCpGpA) which dissociates to a less ordered structure upon raising the temperature. This ordered conformation does not result from an intramolecular rearrangement, as is shown by spectra obtained by varying oligodeoxynucleotide concentration at constant pH. The ordered conformation differs from the Watson–Crick helix, as is shown from nuclear Overhauser enhancement experiments, as well as chemical shift data. An ordered conformation for d(TpCpGpA) was previously reported [Reid, D. G., Salisbury, S. A., Brown, T., & Williams, D. H. (1985) *Biochemistry 24*, 4325–4332]. Their conformation is destroyed upon passing the sample through Chelex [D. H. Williams and J. Kelland, personal communication]; our results indicate that their ordered conformation is similar to the conformation of d(TpCpGpA) we observe between pH 4 and pH 5. In the present case it is likely that stabilization of an ordered duplex conformation for d(TpCpGpA) is achieved by protonation of cytosine. A possible model which could explain the data involves formation of Hoogsteen C^+:G base pairs.

The tetradeoxynucleotide d(TCGA)[1] was reported by Reid et al. (1985) to adopt two conformations that are in slow equilibrium on the NMR time scale at low temperature. ^{1}H NOE studies suggested that both structures were right-handed. The second conformation, predominant at lower temperature, had significant differences from B-DNA. It was proposed that the differences resulted in part from distorted C:G pairing.[2] In the present paper, we present results which show that a proton-stabilized conformation of the oligodeoxynucleotide formed at low temperature when the pH is between 4 and 5 is very similar to the unusual second structure observed by Reid et al. (1985).

It is increasingly evident that pH has substantial influence in determining nucleic acid conformation (Chen, 1984; Htun et al., 1984; Robert-Nicoud et al., 1984; Gray et al., 1984; Brown et al., 1985; Pulleyblank et al., 1985; Antao et al., 1986; Lyamichev et al., 1986; Sarma et al., 1986), particularly for DNA sequences containing cytosine bases. Although the pK_a of cytosine is ~4.5, in polymeric DNA and some oligodeoxynucleotides the pK_a of cytosine can be substantially higher due to the formation of stable conformations involving protonated cytosine. Conformational changes in DNA and oligodeoxynucleotides induced by protonation of cytosine are of considerable current interest because of their possible role in determining higher order DNA structure and conformation and because of the potential for incorporating cytosine-containing oligodeoxynucleotides into DNA cleavage reagents (Moser & Dervan, 1987), which could recognize specific tertiary structure in DNA.

The stable conformation for d(TCGA) between pH 4 and pH 5 is different from that of the Watson–Crick duplex formed at neutral pH. An ordered conformation which involves base pairing of protonated cytosine appears to be formed at the lower pH. This conformation is remarkably stable and is observable in the NMR spectrum at concentrations of oligodeoxynucleotide as low as 70 μM. Below pH 4 a third conformation for d(TCGA) begins to appear in the ^{1}H NMR

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[1] Abbreviations: DNA, deoxyribonucleic acid; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; NOE, nuclear Overhauser effect; ppm, parts per million. Unless otherwise noted, the oligonucleotides discussed in this paper do not have terminal phosphate groups—we abbreviate the nomenclature for oligonucleotides by leaving out the phosphodiester linkage.

[2] The authors now believe that this anomalous conformation is in fact stabilized only under unusual conditions, since they removed it on passing their sample through a Chelex column. The product from the Chelex column showed only a conformation which resembles B-DNA (D. H. Williams and J. Kelland, personal communication).

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spectrum. A possible model for the stable conformation formed between pH 4 and pH 5 is a right-handed duplex in which Hoogsteen C:G base pairs form between protonated cytosine and guanine.

Materials and Methods

Oligodeoxynucleotide Synthesis. The oligodeoxynucleotide d(TCGA) was synthesized with solution-phase phosphotriester methodology (Narang et al., 1979). Standard deblocking procedures were utilized (Sproat & Gait, 1984). The deblocked oligodeoxynucleotide was purified by anion-exchange chromatography (HPLC; strong anion-exchange semipreparative column, elution with an ammonium acetate gradient in 20% ethanol, or low-pressure chromatography; DE-52 anion-exchange resin, elution with a triethylammonium bicarbonate gradient) and reverse-phase chromatography (HPLC; C-18 semipreparative column, elution with acetonitrile). The purified samples were treated with either EDTA or Chelex resin (Bio-Rad) to remove any traces of metal ion impurities. Samples were converted to the sodium salt form and desalted with either gel filtration chromatography (Bio-Gel P-2) or C-18 cartridges (SPICE cartridges, Analtech). The extinction coefficient (ϵ_{260}) utilized for d(TCGA) was calculated to be 40 500 L M^{-1} cm^{-1} (Borer, 1975).

NMR Spectroscopy. The desired quantity of oligodeoxynucleotide was added to 400- or 500-μL aliquots of NMR buffer (0.01 M sodium phosphate, 5×10^{-5} M NaEDTA, 0.1 M sodium chloride, pH 7.0). Samples were then lyophilized from D_2O three times and suspended in 400 μL of 99.998% D_2O for NMR spectroscopy. pH titrations utilized 500 μL of unbuffered samples, which included 100 mM NaCl. Small aliquots of concentrated DCl or NaOD in D_2O were titrated into the NMR sample, accompanied by an aliquot of a stock d(TCGA) solution to maintain the oligodeoxynucleotide concentration. The pH of NMR samples was monitored directly with a small-diameter pH probe (Ingold). pH values in this paper refer to readings from NMR samples in D_2O taken directly from a pH meter standardized from H_2O buffer solutions. These may be correlated with pD values according to the method of Glasoe and Long (1960). To check the concentration dependence of the pH-dependent equilibrium, samples were prepared in buffer of higher ionic strength (0.5 M sodium phosphate, 5×10^{-5} M NaEDTA, 0.1 M sodium chloride).

Results

Observation of Slow Exchange between Conformational States of d(TCGA) at Acidic pH and Low Temperature. A comparison between the 5 °C ^{1}H NMR spectrum of d(TCGA) at pH 7.2 and that at pH 4.2 is shown in Figure 1. At pH 7.2, the NMR spectrum of d(TCGA) exhibits a single set of resonances for the various oligodeoxynucleotide protons (Figure 1A). Upon lowering the pH to 4.2, a spectrum of d(TCGA) very similar to that reported by Reid et al. (1985) is obtained. The pH 4.2 spectrum is shown in Figure 1B.[3] In the pH 4.2 spectrum at 5 °C, two conformations of d(TCGA) are in slow exchange on the NMR time scale. The two conformations

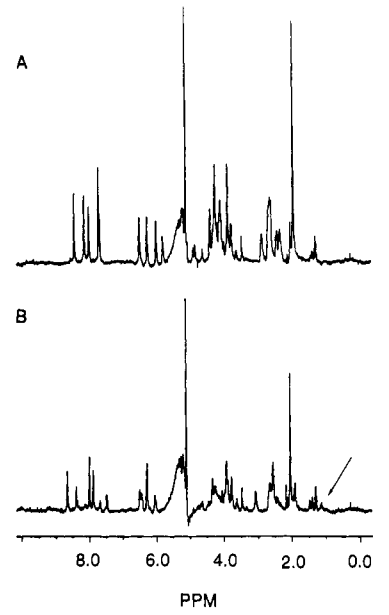

FIGURE 1: ^{1}H NMR spectrum of the tetradeoxynucleotide d(TCGA) at 5 °C, D_2O, and (A) 0.1 M NaCl, pH 7.2, and (B) 0.1 M NaCl, pH 4.2. The spectrum at pH 4.2 closely reproduces that reported previously by Reid et al. (1985). The arrow denotes the location of the cytosine deoxyribose H2′ proton, which has been shifted upfield.

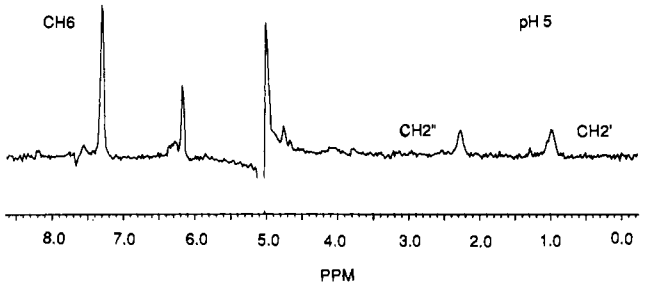

FIGURE 2: Cross section from the 300-MHz ^{1}H NOESY spectrum of d(TCGA) in D_2O at 5 °C. The mixing time was 400 ms. Note the unusual upfield shift for the cytosine H2′ proton, observed at ~1 ppm. A similar upfield shift for this proton was previously noted by Reid et al. (1985).

observed in the spectrum are in unequal proportions; the amount of the minor component increases upon further reduction of pH. As compared to the pH 7.2 spectrum of d(TCGA), substantial chemical shift changes are observed for several protons in the major conformation observed in the pH 4.2 spectrum of d(TCGA); these changes agree with those previously observed by Reid et al. (1985). The cytidine H2′ resonance (denoted by the arrow in Figure 1B) is observed at ~1 ppm, which is at unusually high field. This may be observed clearly in the cytosine H6 cross section through the 5 °C ^{1}H NOESY spectrum of d(TCGA) at pH 5, shown in Figure 2. Likewise, the cytosine H6 proton shifts toward higher field, while the guanine H8, thymine H6, and thymine CH_3 protons shift downfield. The conformation of d(TCGA) at pH 4.2 is different than the Watson–Crick duplex formed at pH 7.2; this may be concluded from the anomalous chemical shifts observed at pH 4.2, and from results of ^{1}H NOE studies. Further evidence supporting an alternative conformation for d(TCGA) at pH 4.2 comes from an investigation of the NMR spectrum as a function of pH.

Chemical Shift Changes Observed for d(TCGA) as a Function of pH at 25 °C. The variations in ^{1}H NMR chemical shifts of the aromatic and anomeric protons of d(TCGA), as a function of pH at 25 °C, are shown in Figure 3. At pH 6.5 and above, the chemical shifts for d(TCGA) are inde-

[3] The earlier workers (Reid et al., 1985) reported the pH of their samples was between 6.8 and 7.2 but have not successfully reproduced their result with Chelex-treated samples under thes conditions (D. H. Williams and J. Kelland, personal communication). Likewise, despite repeated efforts, we have not observed slow equilibrium for d(TCGA) in Chelex- or EDTA-treated samples at neutral pH. Our results indicate that the conformation of d(TCGA) stable at pH 4.2 is very similar to that observed by Reid et al. (1985).

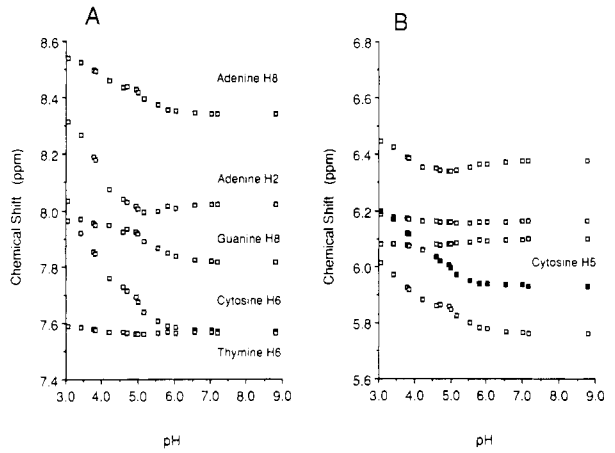

FIGURE 3: pH dependence of ^1H NMR chemical shifts of (A) the base and (B) the cytosine H5 and anomeric protons of d(TCGA) at 25 °C; $[d(TCGA)]_{total}$ = 1 mM. The titration was performed by addition of aliquots of DCl in D_2O to the NMR sample. Above pH 6.5 the chemical shifts are independent of pH, but as the pH is lowered below pH 6.5 significant chemical shifts are observed for the nucleotide base and two of the deoxyribose anomeric protons. The anomeric protons are not assigned.

pendent of pH. This is consistent with the notion that, over this range of pH values at 25 °C, this oligodeoxynucleotide assumes a random-coil configuration. In contrast, as the pH is lowered to form an acidic solution at 25 °C, dramatic chemical shift changes are observed for all but the thymine H6 proton and two of the anomeric proton resonances. The aromatic protons become less shielded at lower pH, with the exception of the adenine H2 proton which becomes somewhat more shielded at intermediate values of pH (pH 4.5–6). The pH titration was not extended below pH 3.2 so as to avoid depurination. Distinct pH titration curves are observable for the adenine H8, guanine H8, and cytosine H6 protons, as well as two anomeric protons and cytosine H5.

The changes induced upon lowering the pH do not correspond to decomposition of the DNA since the original spectrum is reproduced upon readjusting the pH to neutrality. Thus, the observed spectral changes represent a reversible conformational change for the oligodeoxynucleotide as a function of pH. The observation of a titration curve suggests that specific conformational states are stabilized over specific pH ranges; between these pH ranges, equilibrium mixtures of two states are present. In all cases at 25 °C, only a single set of resonances is observed between pH 3 and pH 9; this indicates that the various conformational microstates of the DNA are in rapid exchange on the NMR time scale. Under these conditions, the observed chemical shift at each pH represents the population-weighted average of all microstates available to the oligodeoxynucleotide.

Chemical Shift Changes Observed for d(TCGA) as a Function of pH at 5 °C. Figure 4 shows the results of a pH titration of d(TCGA) at 5 °C. Above pH 7, the spectrum of the base protons is independent of pH, as was observed at 25 °C. At low temperature the rates of exchange among some of the various species present below pH 7 are slowed, and in contrast to the 25 °C data, distinct conformational states (most likely groups of conformationally distinct microstates) are observed. These distinct groups of conformational microstates are labeled I, II, and III in Figure 4. The observed spectra of d(TCGA) as a function of pH at low temperature correspond to distinct regions of the titration curve observed at 25 °C in Figure 3. The precise identities of the conformational states in slow exchange are not known, although as will be discussed the data suggest that conformational state I is a

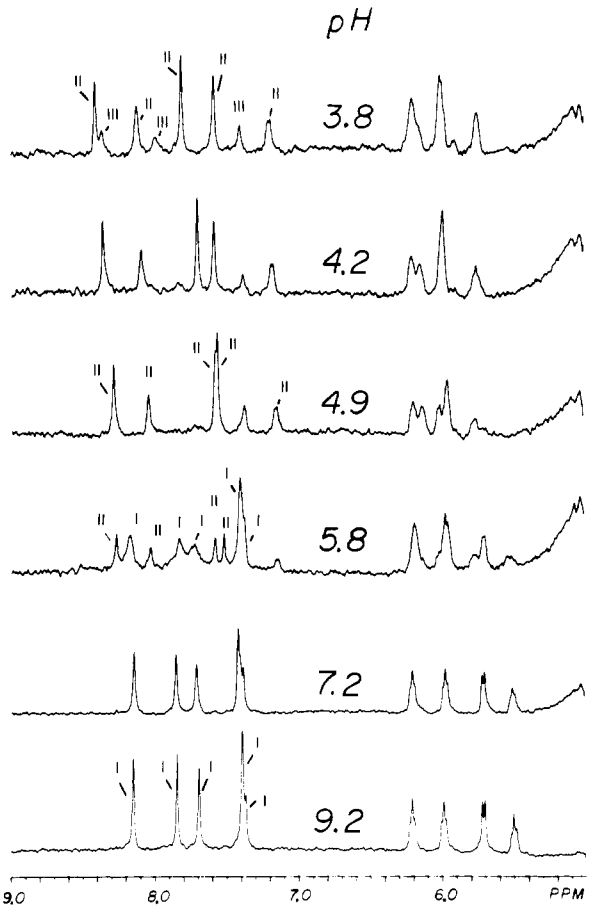

FIGURE 4: pH dependence ^1H NMR chemical shifts at 5 °C; $[d(TCGA)]_{total}$ = 1.0 mM. The titration was performed as described in Figure 3. At the lower temperature, the equilibrium between conformational states is slow on the NMR time scale; distinct sets of resonances are observed as a function of pH. The signals labeled I are assigned to the conformational state present above pH 7; those labeled II are assigned to the low-temperature conformational state stable between pH 4.3 and pH 5; those labeled III are assigned to a third conformational state stable at pH <4. The line width of conformational state II signals is independent of pH while signals from conformational state I increase in line width at lower pH. The assignment of conformational state III signals may be verified by inspection of Figure 6, which shows that as temperature is increased at pH 3.8 conformational state III is favored.

mixture of random coil and the usual Watson–Crick form of d(TCGA) whereas conformational state II is an ordered duplex structure different than the Watson–Crick form.

At pH 7 and above a single set of resonances is observed, corresponding to the adenine H8 and H2 singlets, the guanine H8 singlet, the thymine H6 singlet, the cytosine H5 and H6 doublets, and four pseudotriplets arising from the four anomeric protons of the tetramer. These resonances are assigned as arising from conformational state I. At pH 5.8, a doubling of the observed resonances is seen, assigned as an equilibrium mixture of conformational states I and II. As the pH is lowered to 4.9, the observed spectrum is again dominated by a single set of microstates, labeled as II. This conformational state appears similar to that observed by Reid et al. (1985). Below pH 4.5, the spectrum reflects an equilibrium mixture of conformational states II and III.

Figure 5 shows the influence of pH on the thymine methyl proton resonances for the various species exhibiting slow exchange at 5 °C. These data represent a shifting of the equilibria between conformational states I and II in the pH range between 4.5 and 8.1, favoring conformational state II at low pH. Although the two states are in slow exchange with

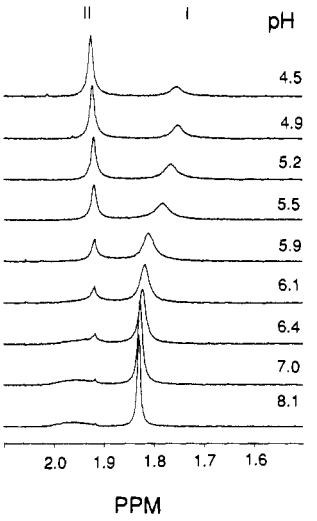

FIGURE 5: Expansion of the thymine methyl region of the ^1H NMR spectrum as a function of pH at 5 °C; $[d(TCGA)]_{total}$ = 6.5 mM. Two conformationally distinct groups of species, labeled conformational states I and II, are in slow exchange under these conditions; conformational state I predominates at pH 7 and above whereas at lower pH conformational state II is the stable species. The line width of the CH_3 resonance of conformational state II (at half-height) is independent of pH, whereas the corresponding resonance for conformational state I is broadened at lower pH. The dissociation rate II → I is independent of pH, whereas the association rate I → II increases as pH is lowered.

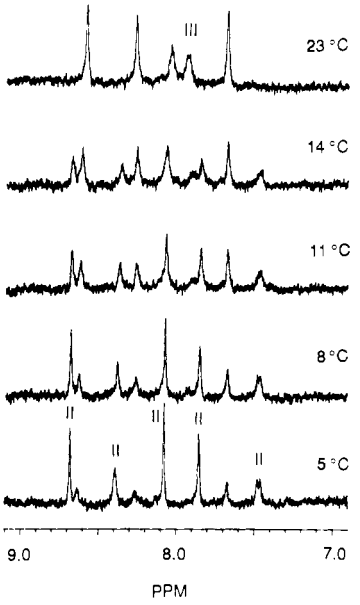

FIGURE 6: Temperature dependence of the equilibrium between conformational states II and III of d(TCGA) at pH 3.8; $[d(TCGA)]_{total}$ = 1.0 mM. Conformational states II and III of d(TCGA) are in slow equilibrium on the NMR time scale at 5 °C. Conformational state II is stabilized at low temperature, but as temperature is raised to 23 °C, conformational state III is favored.

respect to each other, Figure 5 shows that both states exhibit chemical shifts which are dependent on the pH (the dependence is much greater for conformational state I), which indicates that they are not single species but rather a group of conformationally related species which are in rapid exchange on the NMR time scale. Within each group of conformationally related species, the position of the equilibrium depends on the pH of the sample.

Temperature Dependence of Spectra at Constant pH. Figure 6 shows that the equilibrium between conformational states II and III at pH 3.8 is affected by temperature. At low temperature, conformational state II is predominant, whereas

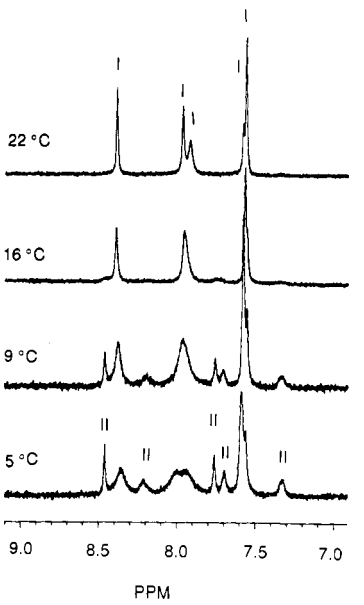

FIGURE 7: Temperature dependence of the equilibrium between conformational states I and II of d(TCGA) at pH 5.9. Conformational states I and II of d(TCGA) are in slow equilibrium on the NMR time scale at 5 °C. Conformational state II is stabilized at low temperature, but as the temperature is raised to 22 °C, conformational state I is favored.

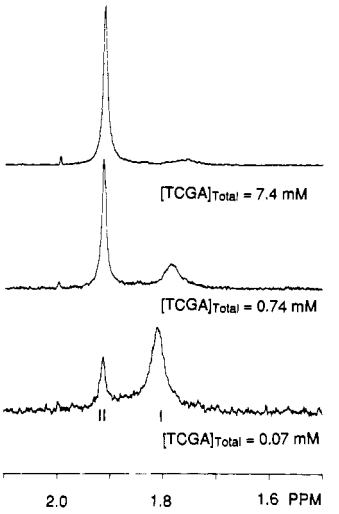

FIGURE 8: Concentration dependence of the equilibrium between conformational states I and II of d(TCGA) at 5 °C and pH 4.7. The figure shows the thymine methyl region of the NMR spectrum. At 7.4 mM strand concentration, conformational state II is favored. At 0.74 mM concentration, a signal for conformational state I appears in the spectrum. If the concentration is dropped to 0.07 mM, the signal corresponding to conformational state I grows in intensity. Note that, even at the lowest concentration examined, a significant amount of conformational state II d(TCGA) exists at this temperature. Inspection of the line widths at half-height for the peaks corresponding to conformational states I and II indicates that the lifetime of conformational state II is independent of concentration, consistent with its removal by the unimolecular dimer → two monomer process, whereas the lifetime of conformational state I is concentration dependent, decreasing at higher concentrations.

at higher temperature conformational state III predominates. A similar result is obtained from the temperature dependence of the spectra at pH 5.9, shown in Figure 7. Under these conditions, conformational states I and II are in equilibrium at 5 °C. As the temperature is raised, the equilibrium shifts to favor conformational state I.

Concentration Dependence of the Equilibrium Involving Conformational States I and II. A series of spectra were obtained as a function of concentration, at pH 4.7 and 5 °C.

The data for the thymine methyl protons are shown in Figure 8, for three samples having concentrations of d(TCGA) of 7.4, 0.74, and 0.07 mM. The equilibrium between conformational states I and II is concentration dependent, and thus not a unimolecular conformational change. Conformational state II is favored at high concentration, although at 0.07 mM concentration a significant proportion of the oligodeoxynucleotide remains in conformational state II. The stability of conformational state II appears to be substantially greater than that of the Watson–Crick duplex which would form at neutral pH; we were unable to drive the equilibrium entirely to the single-stranded state.

Discussion

Evaluation of the ^1H NMR Spectrum of d(TCGA) as a Function of pH. The NMR data are consistent with a model in which d(TCGA) is in the random-coil configuration at room temperature and above, when the pH of the solution is >7. The T_m of d(TCGA) is low under these conditions, but at low temperatures, partial formation of a self-complementary Watson–Crick duplex occurs. This equilibrium is rapid on the NMR time scale, and only a single signal for each proton is observed in the spectrum at all temperatures.

We refer to the pH 7.2 state of d(TCGA) as form a, in which the oligodeoxynucleotide is nonprotonated. Below pH 7, a complex set of conformational equilibria exist, dependent upon oligodeoxynucleotide concentration, temperature, and pH. We refer to the singly protonated state of d(TCGA) as form b and the doubly protonated state as form c. The existence of forms a, b, and c may be inferred from Figure 3. At pH 7 and above d(TCGA) exists in form a. As the pH is lowered, form b appears. Form b is the predominant protonation state between pH 4 and pH 5. Below pH 4, form c appears. Conditions under which form c is predominant were not achieved in the experiment shown in Figure 3, which was not extended below pH 3.2.

Analysis of Conformational States I, II, and III. The existence of conformational states I, II, and III of d(TCGA) appears to be a consequence of the ability of protonation states a, b, and c to form distinct conformations. The energy barrier between conformational states I, II, and III is sufficiently high that the rate of exchange between them is kinetically slow at low temperature. It is important to note that conformational states I, II, and III do not represent unique structures; instead, they represent families of "microstates" which may be defined as sets of unique conformations separated by low-energy barriers such that rapid interconverion (on the NMR time scale) occurs between the various individual conformations of which each family is comprised.

Figure 5 details the behavior of the thymine methyl protons of d(TCGA) at 5 °C as a function of pH. Above pH 7, the signal labeled conformational state I consists of a mixture of form a single-stranded d(TCGA) and the Watson–Crick duplex $d(TCGA)_2$ created by bimolecular association of two form a strands. The relative amount of Watson–Crick $d(TCGA)_2$ is temperature and concentration dependent; Watson–Crick $d(TCGA)_2$ has a low T_m value as determined from measurement of chemical shifts for the pH 7.2 sample as a function of temperature. Below pH 7, the chemical shift of the conformational state I resonance moves toward higher field, broadens, and loses intensity. At lower pH, the conformational state I peak reflects a rapid equilibrium between form a and form b d(TCGA), and the loss of intensity corresponds to an increase in intensity of the conformational state II peak.

Conformational state II has a substantially greater degree of order than does conformational state I. We have performed

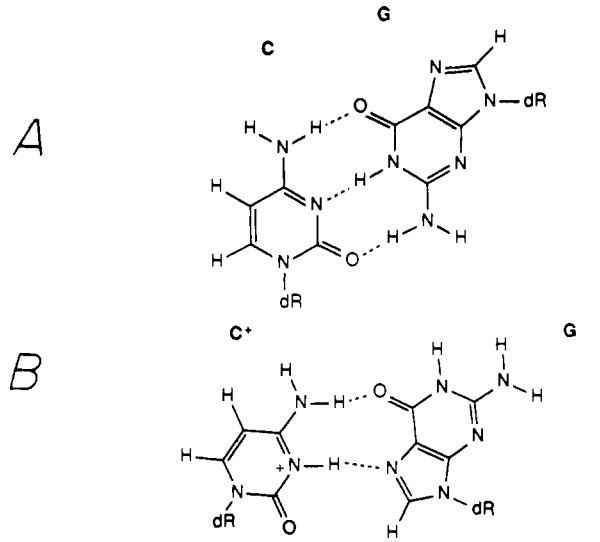

FIGURE 9: Comparison of (A) Watson–Crick C:G base pairing and (B) Hoogsteen C^+:G base pairing.

both 1D and 2D ^1H NOE experiments on a sample of d(TCGA) in conformational state II and have observed NOEs which agree with those observed by Reid et al. (1985), consistent with the notion that conformational state II and the low-temperature conformation previously reported are similar. A cross section from our ^1H NOESY data for the cytosine H6 proton is shown in Figure 2; the remainder of our NOESY data are available as Figure 10 in the supplementary material (see paragraph at end of paper regarding supplementary material). Although these NOEs are in general consistent with a right-handed helix, magnitudes of particular NOEs are significantly different than expected. In particular, the results indicate unusually close contact between AH8 and GH1′, and greater-than-normal distance between GH8 and CH1′ and between AH8 and AH2′H2″, as well as between CH6 and TH2′H2″.

That conformational state II appears at mildly acidic pH indicates that it involves form b d(TCGA). The concentration dependence for the formation of conformational state II (Figure 8) suggests that it is a duplex structure; a similar observation was noted by Reid et al. (1985). The earlier workers also noted the existence of slowly exchanging hydrogen-bonded protons in their sample, presumably arising from interstrand hydrogen bonding. Futhermore, their analysis of the ^{31}P spectrum showed a substantial dispersion of resonances, much greater than would be expected were the oligodeoxynucleotide in the random coil conformation under the conditions of their experiment. Although a higher order associative process cannot be excluded on the basis of the available data, bimolecular association appears to be the most direct explanation for the present observations.

Thus we propose that conformational state II d(TCGA) is a duplex of form b. The most accessible site for protonation is expected to be the N3 position of cytosine. That conformational state II differs from a Watson–Crick duplex is consistent with the observation that the uptake of a proton by cytosine would be expected to disrupt Watson–Crick C:G base pairing. However, if the guanine base were rotated about the glycosidic bond toward the syn conformation, guanine could then form a Hoogsteen base pair with protonated cytosine. Figure 9 shows the standard Watson–Crick C:G base pairing arrangement and an alternative base pairing scheme involving Hoogsteen pairing of protonated cytosine and guanosine. The latter appears to be a reasonable model for conformation state

II d(TCGA) since purine bases are relatively amenable to rotation toward or into the syn conformation.

If guanosine is in the syn conformation, the H8 proton is rotated into close proximity to the deoxyribose H1′ proton and thus should result in a larger NOE between H8 and H1′. The observed NOE between the guanine H8 and H1′ is substantially greater than the NOE between guanine H8 and cytosine H1′, but less than the NOE observed between the H5 and H6 protons of cytidine, which are separated by 2.5 Å. However, quantitative evaluation of this NOE is rendered difficult since a comparison to the magnitude of the guanosine H8–H1′ NOE at pH 7.2 does not provide reliable NOEs. The latter observation is presumably due to the low T_m of the Watson–Crick (form a) duplex $d(TCGA)_2$ which results in a relatively disordered conformation, even at 5 °C. Although Hoogsteen pairing appears to be an attractive model for conformational state II of d(TCGA), it should be considered hypothetical, pending a more detailed analysis of the conformation. It is interesting to note that Reid et al. (1985) reached a similar conclusion in the interpretation of the ^{1}H NOE data generated from their sample, in proposing a rotation of the guanosine about the glycosyl bond into the high-anti conformation.

Although C:G pairs can form Hoogsteen pairing only under conditions in which cytosine is protonated, Hoogsteen pairing between adenine and thymine should be independent of pH in this range, since the thymine N3 position is protonated. Thus, d(TCGA) can potentially form a right-helical structure in which the two C:G pairs are in the Hoogsteen conformation at acid pH or one in which all four base pairs are in the Hoogsteen conformation; the latter configuration would not require formation of a (possibly high-energy) junction between Watson–Crick A:T and Hoogsteen C^+:G pairs.

The stability of conformational state II d(TCGA) is remarkable. Even at pH 7, a trace of the conformational state II thymine methyl peak is observable in the spectrum at 1.92 ppm (Figure 5). Thus, the stabilization induced by formation of conformational state II substantially increases the effective pK_a for cytosine such that a measureable amount of conformational state II is present at neutral pH. The source of this unusual stabilization is likely related to the specific orientation of conformational state II, and warrants further investigation. Reid et al. (1985) hypothesized that the increase in stability noted for their sample may be the result of improved base stacking as compared to the Watson–Crick orientation of the bases.

In summary, conformational state I appears to be a mixture of random coil and Watson–Crick helix formed from bimolecular association of two nonprotonated (form a) d(TCGA) molecules. The random coil consists predominantly of form a d(TCGA) at pH values greater than 7.0, whereas below pH 7.0 random coil form b d(TCGA) is in rapid equilibrium with random coil form a. In contrast, conformational state II is an ordered conformation stable between pH 4 and pH 6 which appears to result from self-association of form b d(TCGA). We have not examined conformation III further, although the data suggest that it may be predominant at high temperature and pH values below 4. The pH dependence of the chemical shifts for d(TCGA) shown in Figure 2 suggests that conformation III results from a second protonation of d(TCGA), possibly at N1 of adenine, generating form c. Note that protonation of adenine at the N1 position precludes formation of Watson–Crick pairing between adenine and thymine.

Protonation of Cytosine. The homopolymers poly(C) and poly(dC) form ordered complexes resulting from self-association at slightly acidic pH. This self-association results in formation of a parallel duplex stabilized by triply hydrogen-bonded hemiprotonated cytosine base pairs. Such base pairing has been observed in the parallel dimer of CpA cocrystallized with proflavin (Westhof & Sundaralingam, 1980). Hemiprotonation of cytosine is also believed to account for the parallel self-association of poly(dCdT) at acid pH (Gray et al., 1980), as well as the oligodeoxynucleotide d(CTCTCT) (Sarma et al., 1986). Brown et al. (1985) demonstrated with photochemical experiments on poly(dCdT) that the formation of cytosine–cytosine dimers, rather than cytosine–thymine dimers, was enhanced at acid pH, suggesting a structure having stacked hemiprotonated $C:C^+$ pairs and looped-out thymidines.

The homopolymer sequences and poly(dCdT) are not capable of forming complementary, Watson–Crick base pairs. Gray et al. (1984) used circular dichroism measurements to examine the conformation of $d(C_4A_4T_4C_4)$ at acid pH. Their results suggested that hemiprotonated $C:C^+$ base pairs would form in the presence of antiparallel Watson–Crick base pairs. Since a three-hydrogen-bond hemiprotonated cytosine base pair in the antiparallel orientation requires that one of the cytosine bases be in the syn conformation about the glycosyl bond, these workers proposed a model in which both cytosines in the hemiprotonated base pair were in the sterically favored anti orientation about the glycosyl bond, and the base pair was stabilized by the presence of two rather than three hydrogen bonds.

Analysis of the Slow Exchange Phenomenon. The observation of slow exchange kinetics between conformational states I, II, and III is unusual. Watson–Crick helix association and dissociation rates measured with T-jump methods for several complementary DNA oligodeoxynucleotides (Nelson & Tinoco, 1982; Freier et al., 1983) indicate relatively rapid helix recombination rates of 10^5–10^6 M^{-1} s^{-1}. Arrhenius plots indicate the activation energy of helix association is low. These results are consistent with the ususal observation of intermediate-to-rapid exchange rates between random coil and Watson–Crick helical states in the ^{1}H NMR spectrum. Insight into the unusual behavior observed for d(TCGA) comes from T-jump experiments on oligodeoxynucleotides helices which are not perfectly complementary, having an unpaired adenine or a G:T mismatch (Chu & Tinoco, 1983). In contrast to results for fully complementary helices, the latter sequences exhibit a substantial activation barrier toward recombination (15–25 kcal/mol). The activation barrier may arise from the particular oligodeoxynucleotide–oligodeoxynucleotide orientation required to initiate duplex formation; helices requiring unusual conformational changes (e.g., formation of Hoogsteen pairing) could have a substantially greater activation barrier.

Other Examples of Slow Conformational Exchange between DNA Structures. For fully self-complementary nucleic acid sequences, rapid exchange precludes the simultaneous observation of singe-stranded and duplexed DNA signals in the ^{1}H NMR spectrum. However, slow exchange has previously been observed in several specific systems. These appear to have the common feature of interchange between two distinct conformational families; each distinct conformational family is presumably represented by a large number of conformational microstates in rapid exchange on the NMR time scale.

One system characterized by a slow exchange process on the NMR time scale is the exchange between duplexes with interior loops, single-stranded species capable of forming hairpin loops. For a series of DNA sequences d(ATCCTA-$[T_n]$TAGGAT), where n = 1–5, when n = 1 only the dimer structure is formed, whereas for n = 3–5 hairpin loop formation is favored. If however, n = 2, then both the dimer

structure and the hairpin loop have approximately equal free energies of formation and are observed in slow exchange on the NMR time scale (Haasnoot et al., 1980). Similar studies on duplex–hairpin transitions in d(CGCGTATACGCG) have been performed (Wemmer et al., 1985).

A second DNA system characterized by slow exchange on the NMR time scale is the B–Z conformational transition observed for $(m^5dC\text{-}dG)_3$ in methanolic solution (Feigon et al., 1984), d(CGm^{5}CGCG) in 1.8 M NaCl buffer (Cavailles et al., 1984), d(m^{5}CGCGm^{5}CG) in 2 M NaCl (Tranh-Dinh et al., 1984), and poly(dA-br^{5}C):poly(dG-T) (Mirau et al., 1986). In these cases both the B and Z conformations can simultaneously be observed in the spectrum. This has allowed the various protons in the Z conformation to be assigned through the utilization of magnetization exchange experiments.

As we were preparing the manuscript, a paper by Manoharan et al. (1987) appeared in which the authors compared two heteroduplexes, d(CGACGC):d(GCGU*GCG) and d(GGCACGG):d(CCGU*GCC), where the deoxyuracil was double labeled with ^{13}C. At 0.1 M NaCl, pH 7, the second of these two heteroduplexes slowly interconverted between two conformations, whereas only a single conformation was observed for the first heteroduplex. The two conformers for the second duplex appear to be right-handed.

Conclusions

Our results indicate that the tetranucleotide d(TCGA) readily forms a remarkably stable ordered conformation between pH 4 and pH 5. We propose that this is a duplex stabilized by protonation of the oligodeoxynucleotide at the N3 position of cytosine. A possible conformation is one in which the two strands are held together by C$^+$:G Hoogsteen base pairing. This complex exhibits greater stability than the corresponding Watson–Crick duplex formed at neutral pH. These results point also to the substantial influence of pH on the conformation of cytosine-containing oligodeoxynucleotides, where the effective pK_a of cytosine may be significantly higher than expected due to the stability of specific conformational states involving protonated cytosine.

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Supplementary Material Available

Figure 10 showing cross sections through the phase-sensitive 300-MHz ^{1}H NOESY spectrum obtained at pH 5 and 5 °C for conformational state II of d(TCGA) for a mixing time of 400 ms, cross sections being plotted for the adenine H8, guanine H8, thymine H6, and cytosine H6 protons of d(TCGA) (1 page). Ordering information is given on any current masthead page.

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