CD evidence that the alternating purine-pyrimidine sequence poly[d(A-C)·d(G-T)], but not poly[d(A-T)·d(A-T)], undergoes an acid-induced transition to a modified secondary conformation[§]

Vincent P. Antao⁺, Robert L. Ratliff¹ and Donald M. Gray^{*}

Program in Molecular and Cell Biology, Mail Station 3.1, University of Texas at Dallas, Box 830688, Richardson, TX 75083-0688 and ¹Life Sciences Division, Mail Stop 886, Los Alamos National Laboratory, Box 1663, Los Alamos, NM 87545, USA

Received January 17, 1990; Revised and Accepted June 19, 1990

ABSTRACT

Circular dichroism and UV absorption data showed that poly[d(A-C)·d(G-T)] (at 0.01M Na⁺(phosphate), 20°C) underwent two reversible conformational transitions upon lowering of the pH. The first transition was complete at about pH 3.9 and resulted in an acid form of the polymer that was most likely a modified, protonated duplex. The second transition occurred between pH 3.9 and 3.4 and consisted of the denaturation of this protonated duplex to the single strands. UV absorption and CD data also showed that the separated poly[d(A-C)] strand formed two acid-induced self-complexes with pK_a values of 6.1 and 4.7 (at 0.01M Na⁺). However, neither one of these poly[d(A-C)] self-complexes was part of the acid-induced rearrangements of the duplex poly[d(A-C)·d(G-T)]. Acid titration of the separated poly[d(G-T)] strand, under similar conditions, did not show the formation of any protonated poly[d(G-T)] self-complexes. In contrast to poly[d(A-C)·d(G-T)], poly[d(A-T)·d(A-T)] underwent only one acid-induced transition, which consisted of the denaturation of the duplex to the single strands, as the pH was lowered from 7 to 3.

INTRODUCTION

Our studies with simple DNA sequences have shown that protonation of single- as well as double-stranded DNA may result in a variety of conformations.¹⁻⁸ CD spectral studies have shown that samples of poly[d(G)·d(C)] upon heat denaturation of the double helix, or upon exposure to acid, undergo rearrangement to give the poly[d(C⁺)·d(C)] self-complex and free poly[d(G)].⁹⁻¹⁰ Our spectral studies have also shown that

the alternating purine-pyrimidine polymer poly[d(G-C)·d(G-C)] undergoes two acid-induced transitions (other than denaturation), each resulting in a structure with distinct CD spectral characteristics.⁵ We have proposed that the first acid form of poly[d(G-C)·d(G-C)] may consist of about 50% protonated G·C⁺ base-pairs, possibly of the ^{syn}G·C⁺ type between antiparallel strands, and that the second form, which probably involves strand rearrangement, most likely consists of C·C⁺ base-pairs alternating with G·G base-pairs between parallel strands.⁵ More recently, we have shown that the acid-induced rearrangements of poly[d(A-G)·d(C-T)] include not only the protonated poly[d(C⁺-T)·d(A-G)·d(C-T)] triplex, but also include the protonated self-complexes of the individual poly[d(C-T)] and poly[d(A-G)] strands.⁶

To enable one to predict whether a particular natural DNA sequence could form such protonated structures, we have now completed a survey of protonated structures formed in simple, repeating DNA sequences. We present here our CD data for the acid-induced transitions of poly[d(A-C)·d(G-T)] and poly[d(A-T)·d(A-T)].

Poly[d(A-C)·d(G-T)] is an alternating purine-pyrimidine sequence that is found quite frequently in eukaryotic DNA sequences.¹¹⁻¹⁴ This sequence has been extensively studied as a model for the B to Z transition.¹⁵⁻²³ From CD spectra obtained during the forward (acid) and back (alkali) titrations of poly[d(A-C)·d(G-T)] (pH 7.0 to 2.8, 0.15M Na⁺, 25°C), Thiele *et al.*²⁴ found that the structural changes were pronounced below pH 4, and that both the forward and back CD titrations were superimposable. Their data might be interpreted as indicating only one acid-induced transition for this polymer. We have performed detailed titrations of the duplex poly[d(A-C)·d(G-T)] and also of the individual strands, poly[d(A-C)] and

* To whom correspondence should be addressed

⁺ Present address: Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, CA 94720, USA

[§] This paper on the investigation of the acid-induced transitions of poly[d(A-C)·d(G-T)] and poly[d(A-T)·d(A-T)] is the third in a series of three papers on CD and UV absorption studies of the acid transitions of DNA sequences that have a simple dinucleotide repeat. The papers on the acid-induced transitions of poly[d(G-C)·d(G-C)] and poly[d(A-G)·d(C-T)] have been published.^{5,6}

poly[d(G-T)], at both 0.01M Na⁺ and 0.1M Na⁺. Our CD and absorption data clearly showed that there were two reversible acid transitions. The first transition led to a conformation that was most likely a protonated duplex, while the second transition consisted of the denaturation of the modified duplex to give the protonated single-strands. Poly[d(A-C)] formed two protonated self-complexes, neither of which was a part of the acid-induced transitions of the duplex poly[d(A-C)·d(G-T)]. Poly[d(G-T)] did not form any unusual protonated structures down to pH 2.6.

Poly[d(A-T)·d(A-T)] is a self-complementary, alternating purine-pyrimidine sequence. The conformational variability of this polymer includes the B, A, and Z conformational families.²⁵ For poly[r(A)] and poly[d(A)], lowering of the pH leads to the formation of self-complexes that have large CD bands (Ref. 26 and our unpublished results). It is also possible that the protonation of the adenine might be responsible for stabilizing the pH-dependent A·C mismatches observed for DNA oligonucleotides²⁷⁻²⁹ and more recently for a RNA hairpin

structure.³⁰ To determine if the protonation of poly[d(A-T)·d(A-T)] might lead to the formation of a protonated structure, (for example, one having a helical core of A·A⁺ base-pairs and the intervening thymidyl residues looped out), we have performed detailed acid titrations of poly[d(A-T)·d(A-T)] at 0.01M and 0.1M Na⁺. Our data showed that the polymer underwent only one transition, which consisted of denaturation of the duplex to the single strands.

MATERIALS AND METHODS

Poly[d(A-C)·d(G-T)] and poly[d(A-T)·d(A-T)] were purchased from Pharmacia P-L Biochemicals. The individual strands, poly[d(A-C)] and poly[d(G-T)], were isolated using alkaline cesium chloride density gradient centrifugation as described by Wells and Blair.³¹ Since complete resolution of the two peaks was not obtained with a single centrifugation, pooled aliquots from each of the two peaks were individually purified by a second

Figure 1. CD spectra of poly[d(A-C)·d(G-T)] at 0.01M Na⁺, 20°C, as the pH was lowered: (A) from 7.0 to 3.9, and (B) from 3.9 to 3.4.

density gradient centrifugation. The polymers were dialyzed first against 0.01M EDTA, 0.5M NaCl, and 0.01M Na⁺(phosphate), second against 0.01M NaCl and 0.01M Na⁺(phosphate), and finally against 0.01M Na⁺(phosphate), all at pH 7. The extinction coefficients at 260nm used for the double-stranded poly[d(A-C)·d(G-T)] and poly[d(A-T)·d(A-T)] were 6500 and 6600 L·mol⁻¹·cm⁻¹, respectively.³²⁻³³ Extinction coefficients at 260nm of 9800 and 10,480 L·mol⁻¹·cm⁻¹ were used for the single-stranded polymers poly[d(A-C)] and poly[d(G-T)], respectively; these were previously calculated using first-neighbor equations.^{1,34}

UV absorption spectra were measured with a Cary-Varian Model 118 spectrophotometer. CD measurements were made using a Jasco J500A circular dichrometer, calibrated as described previously.⁶ Concentrations were determined from the absorbance measurements and the extinction coefficients given above. The digitized CD data were processed as in previous work.⁶ CD values are given as L·mol⁻¹·cm⁻¹. Sample temperatures during the optical measurements were controlled using a Neslab programmable water circulator. The reported temperatures are accurate to within $\pm 0.5^\circ\text{C}$.

Unless otherwise specified, the pH titration of samples was carried out at 20°C in a starting buffer of 0.01M Na⁺(phosphate), pH 7. Aliquots of a 4M NaCl solution were used to increase the Na⁺ concentration to 0.1M Na⁺. The pH was lowered by the addition of small aliquots of dilute HCl and was raised with small aliquots of NaOH solutions. Spectra as a function of pH were corrected for dilution by HCl and NaOH. The total dilution was never more than 7%. The sample pH was measured directly before and after each set of optical measurements. The reported pH values are accurate to within ± 0.05 pH unit.

To determine the extent of depurination during the acid and

back titrations, triplicate samples of poly[d(A-C)·d(G-T)] at 0.01M Na⁺ (phosphate) were subjected to the extremes of pH and temperature encountered in these experiments. The samples were then precipitated with perchloric acid at 0°C and quickly passed through a 0.45 μm Millipore filter. The extent of depurination was determined from the non-precipitable material in the filtrate. A standard for a completely depurinated sample was prepared by heating triplicate samples of poly[d(A-C)·d(G-T)] at pH 2.5 for 2.5h at 90°C. The extent of depurination in these samples was taken as 100%. When compared with this standard, the depurination of samples equivalent to those subjected to the most extreme conditions in these experiments was less than 3%. We have not determined quantitatively an upper limit for the extent of depurination of the poly[d(A-T)·d(A-T)] samples. However, on the basis of the analysis of the poly[d(A-C)·d(G-T)] samples, and of similar results we obtained previously with poly[d(G-C)·d(G-C)] and poly[d(A-G)·d(C-T)],^{5,6} we expect the extent of depurination of our poly[d(A-T)·d(A-T)] samples to also be less than 3%, especially since none of the latter samples were heated at acid pH.

RESULTS

Acid-induced Spectral Transitions of Poly[d(A-C)·d(G-T)]

CD and UV absorption data of poly[d(A-C)·d(G-T)] indicated that two acid-induced transitions occurred as the pH was decreased. The CD spectra characterizing these transitions are shown in Figure 1, and CD changes at specific wavelengths as a function of pH are shown in Figure 2. The first major transition occurred between neutral pH and pH 3.9 (at 0.01M Na⁺). Between pH 7 and 5, there were only minor changes in the magnitudes of the CD bands for the polymer at 0.01M Na⁺ (see Figure 1A), and there were no detectable changes in spectra of the polymer at 0.1M Na⁺ (data not shown). However, below pH 5, the magnitudes of the CD bands at 280, 240, and 223nm decreased. Isodichroic points at 261 and 230nm strongly suggested that only two distinct forms of the polymer were present in the pH range 5 to 3.9 at 0.01M Na⁺ (Figure 1A) and over the pH range 7 to 3.1 at 0.1M Na⁺ (not shown). The UV absorption spectra corresponding to these CD spectral changes were slightly hypochromic at 258nm and hyperchromic above 266nm (Figure 3). The maximum decrease in the magnitudes of the CD bands at 280, 240, and 223nm and the maximum hypochromicity (about 2% at 258nm) were observed at pH 3.9 at 0.01M Na⁺. The polymer at pH 4.0 and 0.01M Na⁺ melted cooperatively with a T_m of 39°C; the absorption spectrum of the melted polymer at 45°C is shown in Figure 3. At neutral pH, the T_m of poly[d(A-C)·d(G-T)] at 0.01M Na⁺ is 65.5°C.³⁵

Our CD spectral data indicated that a second acid transition occurred between pH 3.9 and pH 3.4 (see Figures 1 and 2). The CD changes were characterized by an increase in the positive CD band at 281nm, a reversal of the sign of the CD at 260nm, and a decrease in the magnitude of the negative CD at 240nm (Figure 1B). To within the error of measurement, isodichroic points were observed at 271, 253, and 230nm, making it likely that there was only one transition in this pH range. The UV absorption spectra over this small pH range (pH 3.9 to 3.4) showed increasing hyperchromicity, and at pH 3.4 the spectrum was about as hyperchromic as that due to melting the polymer at pH 4.0 (Figure 3).

Both of these acid-induced transitions were almost completely reversed upon back titration with NaOH.

Figure 2. Acid titration of poly[d(A-C)·d(G-T)] at 0.01M Na⁺ as monitored by the CD at 280nm (●), 265nm (▲), and 240nm (■).

Figure 3. UV absorption spectra of poly[d(A-C)·d(G-T)] at 0.01M Na⁺: at pH 7.0, 20°C (—); at pH 3.9, 20°C (+); at pH 3.4, 20°C (---); and at pH 4.0, 45°C (*).

UV absorption and CD spectral changes similar to those described above for poly[d(A-C)·d(G-T)] at 0.01M Na⁺ were also observed at the higher salt concentration of 0.1M Na⁺ (0.01M Na⁺(phosphate) + 0.09M NaCl), 20°C. However, the pK_a of each of the transitions of the polymer at 0.1M Na⁺ was about 0.75 pH unit lower than the pK_a of the corresponding transition at 0.01M Na⁺.

Acid-induced Spectral Transitions of Poly[d(A-C)]

UV absorption and CD data of poly[d(A-C)] at 0.01M Na⁺ indicated that three acid-induced transitions occurred as the pH was decreased. As shown in the inset to Figure 4, these transitions were rather sharp, taking place over narrow pH ranges (<0.6 pH unit). The first transition, which occurred between pH 6.3 and 5.8, was characterized by a large increase in the positive CD near 270nm, accompanied by a relatively modest increase in the negative CD at 247nm (Figure 4). The UV absorption spectrum at pH 5.5 was hypochromic below 284nm (about 20% at 260nm), and hyperchromic above 284nm, relative to the spectrum at pH 7.2 (Figure 5). This form of poly[d(A-C)] (at pH 5.5, 0.01M Na⁺) melted cooperatively with a T_m of 33°C (data not shown). These data indicated that poly[d(A-C)] existed in an ordered, stacked structure at pH 5.5.

The second acid-induced transition occurred as the pH was

lowered from 5.0 to 4.4 (see inset to Figure 4). The CD spectrum of the second acid form of poly[d(A-C)] had a very broad positive band that extended from 258 to 310nm. The UV absorption spectrum showed increases at all wavelengths between 310 and 210nm (Figure 5). The extinction coefficient at 260nm of the second acid form (at pH 4.4) was about 4% greater than that of the first acid form (at pH 5.5), but was still about 16% less than that of the single-stranded form at neutral pH. The second acid form (at pH 4.5, 0.01M Na⁺) melted cooperatively, with a T_m of about 28°C (data not shown).

These two pH-dependent transitions were also observed for poly[d(A-C)] at the higher salt concentration of 0.1M Na⁺. The pK_a values for the first transition were very similar at both 0.01 and 0.1M Na⁺, but the pK_a value for the second transition of the polymer at 0.1M Na⁺ was about 0.3 pH unit lower than that measured at 0.01M Na⁺.

The third transition was observed upon titrating poly[d(A-C)] at 0.01M Na⁺ to pH values below 4 and consisted of the denaturation of the second acid self-complex. This was evidenced by large decreases in the intensities of the major CD bands (Figure 4), giving rise to spectral properties similar to those of the heat-denatured poly[d(A-C)] at low pH (data not shown). The UV absorption increased at wavelengths above 256 and below 242nm during this transition (Figure 5). At 0.1M Na⁺, and to a lesser

Figure 4. CD spectra of poly[d(A-C)] at 0.01M Na⁺, 20°C: at pH 7.2 (— — —); at pH 5.5 (×); at pH 4.5 (○); and at pH 3.2 (+). Inset: the acid titration of poly[d(A-C)] as monitored by the CD at 290nm (●) and 270nm (▲).

extent at 0.01M Na⁺, precipitation of the polymer began to occur before denaturation was complete.

Acid Titration of Poly[d(G-T)]

The CD spectral changes observed for poly[d(G-T)] at 0.01M Na⁺, 20°C, as the pH was decreased to pH 2.9 are shown in Figure 6. The CD bands at 282 and 258nm decreased in magnitude and shifted to longer wavelengths as the pH was lowered from neutral pH to 2.9 (Figure 6) or to 2.6 (inset to Figure 6). The magnitudes of these CD bands continuously decrease in spectra of poly[d(G-T)] at neutral pH as the temperature is increased from 20 to 90°C, presumably as a result of decreased base stacking in the single-stranded polymer.³⁴ Therefore, we believe that the spectral changes shown in Figure 6 were the result of changes in the base stacking of single-stranded poly[d(G-T)], possibly coinciding with protonation of the

guanines, as the pH was reduced. The CD spectral changes observed during the acid titration of poly[d(G-T)] at the higher ionic strength of 0.1M Na⁺ were similar to those seen for the polymer at 0.01M Na⁺, although the magnitudes of the CD bands were less for the polymer at the higher salt concentration.

Acid-induced Spectral Transition of Poly[d(A-T)·d(A-T)]

CD and UV absorption data of poly[d(A-T)·d(A-T)] indicated that only one major acid-induced transition occurred as the pH was decreased from neutral pH to pH 3. As shown in the inset to Figure 7, the transition was a sharp one, occurring over a small range of pH (pH 4.8 to pH 4.2 at 0.01M Na⁺). Between neutral pH and pH 4.8, there were only minor changes in the CD spectra of the polymer. Below pH 4.8, the magnitudes of the CD bands at 262, 247, and 225nm decreased. The intensity of the CD at wavelengths longer than 268nm increased, giving rise to a distinct

Figure 5. UV absorption spectra of poly[d(A-C)] at 0.01M Na⁺, 20°C; at pH 7.2 (---); at pH 5.5 (×); at pH 4.5 (○); and at pH 3.2 (+).

positive CD band at 272nm. Isodichroic points at 236, 250, and 267nm indicated that only two distinct forms of the polymer were present in the pH range 7 to 4.2 (Figure 7). The sample exhibited only minor CD spectral changes below pH 4.2.

The acid-induced transition observed for poly[d(A-T)·d(A-T)] was reversed upon back titration with NaOH.

The UV absorption spectra corresponding to the CD spectral changes described above showed only minor changes between neutral pH and pH 4.8 (data not shown). Below pH 4.8, the polymer was hyperchromic with respect to the polymer at neutral pH at all wavelengths between 210 and 300nm. The hyperchromicity at 260nm for the polymer in the pH range 4.2 to 3 (at 0.01M Na⁺), with respect to the polymer at neutral pH, was greater than 50%, consistent with that observed upon melting poly[d(A-T)·d(A-T)] at neutral pH.³⁶ The protonation of the dAMP monomer causes only small changes in the UV absorption spectrum under similar conditions, with little, if any, hyperchromicity at 260nm.³⁷ These data showed that the acid-

induced transition for poly[d(A-T)·d(A-T)] consisted of denaturation of the duplex polymer.

UV absorption and CD spectral changes similar to those observed for the polymer at 0.01M Na⁺ were also observed for the polymer at 0.1M Na⁺ (0.01M Na⁺(phosphate) + 0.09M NaCl), 20°C (data not shown). However, the pK_a of the transition for the polymer at 0.1M Na⁺ was about 1.1 pH unit lower than that for the polymer at 0.01M Na⁺.

DISCUSSION

First Acid Transition of Poly[d(A-C)·d(G-T)]

The changes in the UV absorption and CD spectra for poly[d(A-C)·d(G-T)] indicated that the first acid transition was a broad one occurring over a range of more than two pH units. Although the CD bands decreased in magnitude during this transition, the CD spectra increased in complexity, as evidenced by a broadening

Figure 6. CD spectra of poly[d(G-T)] at 0.01M Na⁺, 20°C: at pH 7.2 (— — —); at pH 4.6 (×); at pH 4.0 (○); at pH 3.4 (+); and at pH 2.9 (*). Inset: the acid titration of poly [d(G-T)] as monitored by the CD at 280nm (●) and 260nm (▲).

of the long-wavelength positive CD band and an indication that the CD above 258nm may be composed of three bands (Figure 1A). The UV absorption at 260nm did not markedly change upon acid titration to pH 3.9 (Figure 3), suggesting that there was little or no unstacking of the bases or strand separation. The increased absorption at longer wavelengths (>280nm) was consistent with the protonation of the cytosine residues of the polymer.³⁷ The three possible sites of protonation were the N3 of cytosine (pKa of 4.3 and 4.6 for dC and 5'-dCMP, respectively), the N1 of adenine (pKa of 3.8 and 4.4 for dA and 5'-dAMP, respectively), and the N7 of guanine (pKa of 2.5 and 2.9 for dG and 5'-dGMP, respectively).³⁸ The pKa values of these bases in double-stranded calf-thymus DNA tend to be slightly lower than those cited for the monomers; however, the order of protonation remains the same as for the monomers.^{39,40} Also, in the case of free or cloned [d(A-G)·d(C-T)]_n sequences, cytosine is the first base to become protonated as the pH is reduced, leading to the formation of a triplex.^{6,41,42} At 0.01M Na⁺, while poly[d(A-G)·d(C-T)] forms a triplex at pH 6–7, protonation of the bases in the poly[d(A-G)] strand to form a self-complex occurs only below pH 5.5.⁶

None of the CD spectra measured during the first acid-induced transition of poly[d(A-C)·d(G-T)] matched the average of spectra of poly[d(A-C)] and poly[d(G-T)] measured individually, at acid pH values (Figure 8A). This showed that the first acid-induced transition of the duplex did not involve denaturation to the single-strands, or the formation of either of the protonated self-complexes of poly[d(A-C)]. On the basis of the data presented here, it appeared that protons were taken up by the polymer

without strand separation, and that the first acid form of poly[d(A-C)·d(G-T)] was a modified, protonated duplex.

Protonation of the cytosines would disrupt the normal Watson-Crick G·C base-pairs. Three possible arrangements for protonated G·C⁺ base-pairs are the Hoogsteen, reversed Hoogsteen, and slipped Watson-Crick base-pairs.⁴³ Since the pKa of adenine is lower than that of cytosine even in double-stranded DNA,^{39,40} it is not necessary to postulate the presence of non-Watson-Crick A·T base-pairs in the first acid form of poly[d(A-C)·d(G-T)]. Thus, the first acid conformation of poly[d(A-C)·d(G-T)] might consist of protonated G·C⁺ base-pairs, alternating with Watson-Crick A·T base-pairs.

Second Acid Transition of Poly[d(A-C)·d(G-T)]

During the second acid transition, the spectral changes included an increase in the magnitude of the positive CD at 283nm, a reversal of the sign of the CD at 260nm, and an increase in the UV absorption (Figures 1B and 3). The CD spectrum measured for poly[d(A-C)·d(G-T)] at pH 3.4 at the end of the second transition agreed reasonably well with that measured for the melted polymer at pH 4.0, 45°C, and there was even better agreement with a simple average of the spectra measured separately at 20°C for poly[d(A-C)] and for poly[d(G-T)] at pH 2.9 (Figure 8B). Thus, these data indicated that the second acid transition consisted of denaturation of the first acid form to the protonated single-strands. This denaturation could be brought about by lowering the pH (from 3.9 to 3.4) or by increasing the temperature (for example, to 45°C at pH 4.0). In both cases, the denaturation was reversible with no observed hysteresis.

Figure 7. CD spectra of poly[d(A-T)·d(A-T)] at 0.01M Na⁺, 20°C: at pH 6.9 (— — —); at pH 4.8 (●); at pH 4.5 (+); and at pH 4.2 (*). Inset: the acid titration of poly[d(A-T)·d(A-T)] as monitored by the CD at 262nm (▲) and 247nm (■).

Many of the spectral changes observed during the second acid transition differed from those observed during the first transition, indicating that denaturation was not the primary result of protonation of poly[d(A-C)·d(G-T)] during the first transition. For example, (1) the positive CD band at 280nm decreased during the first transition, but increased during the second transition (Figure 1), and (2) the UV absorption at 260nm showed a very small hypochromicity during the first transition, but was hyperchromic during the second transition (Figure 3).

Two Acid-induced Self-complexes of Poly[d(A-C)]

Poly[d(A-C)] at neutral pH is in a single-stranded form.¹ However, lowering the pH in small decrements revealed three cooperative transitions that each occurred over a narrow pH range (<0.6 pH unit). The first two transitions resulted in forms of the polymer that were hypochromic at 260nm and hyperchromic above 285nm (or in the case of the second form, above 273nm),

when compared with the single-stranded form at neutral pH (Figure 5).

There are several possibilities for the type of base-pairing that could exist in a protonated poly[d(A-C)] self-complex: (1) C·C⁺ base-pairs could form as in the self-complexes of poly[d(C)] and poly[d(C-T)]. The adenines could then be extrahelical or intrahelical, paired or unpaired. (2) A⁺·A⁺ base-pairs could form as in the acid-induced poly[d(A)] self-complex.²⁶ (3) Protonated A·C base-pairs could also form. There are several types of protonated A·C base-pairs involving the major tautomeric forms of the bases that could exist between antiparallel strands.^{1,27} The A·C base-pair observed in the crystal structure of the dodecamer d(CGCAAATTCGCG)·d(CGCAAATTCGCG) shows one close approach between the N6 amino group of adenine and the N3 of cytosine, and another short distance between two electron acceptor groups, the N1 of adenine and the O2 of cytosine.²⁷ In the latter case,

Figure 8. A comparison of calculated and measured CD spectra of poly[d(A-C)·d(G-T)]. (A) The spectrum of poly[d(A-C)·d(G-T)] measured at the end of the first acid transition (pH 3.9, 20°C), is compared with the simple averages of spectra of poly[d(G-T)] and of poly[d(A-C)] at three acid pH values. (B) The CD spectrum measured for poly[d(A-C)·d(G-T)], at pH 3.4, 20°C, is compared with the measured spectrum of the denatured poly[d(A-C)·d(G-T)] at pH 4.0, 45°C, and with the simple average of spectra of the individual strands at low pH.

protonation at the N1 of adenine could result in a hydrogen bond that holds the two acceptor groups in close proximity.²⁷ NMR data of other oligodeoxyribonucleotides with A·C mismatches are consistent with this type of base-pairing.^{28,29,44} An A·C base-pair could also conceivably form with the added proton shared between the N3 of cytosine and the N1 of adenine, with a second hydrogen bond formed between the O2 of cytosine and the amino group of adenine, again leading to a uniform structure, but with parallel strands if the N-glycosyl bonds remain *anti*.

First Acid Form of Poly[d(A-C)]

The first acid-induced self-complex of poly[d(A-C)] was characterized by a large positive CD band at 269nm and a negative CD band at 247nm (Figure 4), and exhibited about 20% hypochromicity at 260nm when compared with the single-stranded form at neutral pH (Figure 5). The increase in the absorption at wavelengths above 284nm, together with the small but significant increase in CD above 290nm, indicated that the cytosines were protonated (or shared a proton at N3).³⁷

However, it seems unlikely that stacked $C \cdot C^+$ base-pairs were involved in this self-complex of poly[d(A-C)], since the increase in the CD above 290nm was very small compared with that observed upon formation of stacked $C \cdot C^+$ base-pairs in poly[d(C)], the loop-out structure of poly[d(C-T)] (in which thymidyl residues are looped-out of a core of stacked $C \cdot C^+$ base-pairs), or oligomers containing stretches of cytosines.^{3,7,8} The existence of stacked $A^+ \cdot A^+$ base-pairs might also be excluded as the only type of base-pair, since the formation of $A^+ \cdot A^+$ base-pairs in poly[d(A)] results in a large positive CD band at 260nm (our unpublished results), as opposed to 270nm for this poly[d(A-C)] self-complex. Also, poly[d(A)] becomes protonated with a pKa of 4.4 in 0.1 M NaCl,²⁶ much lower than the pKa of 6.1 for the first acid-induced transition of poly[d(A-C)]. Furthermore, under similar conditions, there was no evidence for a protonated structure with $A^+ \cdot A^+$ base-pairs for poly[d(A-T)·d(A-T)] at low pH. Thus, the remaining possibilities for base-pairing in this first acid form of poly[d(A-C)] appeared to be protonated A·C base-pairs, or perhaps protonated $C \cdot C^+$ base-pairs with intrahelical adenines (i.e. without the cytosines being stacked on top of each other as in the loop-out structure of poly[d(C-T)]^{1,3,4}).

Second Acid Self-complex of Poly[d(A-C)]

The CD spectrum of the second poly[d(A-C)] self-complex, characterized by a large, rather broad positive band extending from 310 to about 260nm, and a small negative band from 238 to 260nm (Figure 4), was similar to that previously reported for [d(A-C)]₆₇ at 0.07M Na⁺, pH 4, 20°C.¹ The UV absorption spectrum corresponding to this CD spectrum showed that this second acid form of poly[d(A-C)] was hyperchromic compared with the first acid form (Figure 5), suggesting that the bases in the second acid form may not be as well stacked as in the first acid form.

The large CD band extending to 310nm strongly suggested that $C \cdot C^+$ base-pairs were a part of this protonated self-complex. The CD spectrum of this protonated self-complex did not agree well with one calculated for a simple loop-out structure in which the intervening adenyl residues were non-interacting and looped out of a helix stabilized by $C \cdot C^+$ base-pairs.¹ Thus, if the cytosines were paired, the adenines interacted in some way, either intrahelically or extrahelically.

It is interesting that the CD spectra from detailed titrations of poly[d(A-C)·d(G-T)] at both 0.01M Na⁺ and 0.1M Na⁺ gave no indication of the presence of either protonated self-complex of poly[d(A-C)]. Even disrupting the duplex at acid pH values, by heating the samples, did not lead to the formation of either protonated self-complexes of poly[d(A-C)] upon reannealing (data not shown). This can be contrasted to our earlier finding that the pH-induced structural rearrangements of the polypurine·polypyrimidine sequence poly[d(A-G)·d(C-T)] included the protonated self-complexes of the individual strands poly[d(A-G)] and poly[d(C-T)], if the duplex or triplex forms were first disrupted by decreasing the pH or by increasing the temperature.⁶ To confirm that the poly[d(A-C)] self-complexes were not stable in the presence of free poly[d(G-T)], equimolar mixtures were made of poly[d(G-T)] and of each of the poly[d(A-C)] self-complexes (at pH 5.5 and 4.4). CD spectra measured about a half hour after making the mixture, showed that most of the first poly[d(A-C)] self-complex was still present in the mixture at pH 5.5, while only a small portion of the second poly[d(A-C)] self-complex was present in the mixture at pH 4.4.

However, CD spectra measured a few days later were similar to those measured for the duplex polymer at pH 5.5 and pH 4.4, thus showing that the protonated duplex was a rearrangement that was favored over one that would involve either of the poly[d(A-C)] self-complexes co-existing with free poly[d(G-T)].

Although the types of base-pairing in the protonated forms of poly[d(A-C)] remain to be determined, the finding that poly[d(A-C)] existed in two protonated self-complexes is an important one since this is the first simple DNA sequence (with non-complementary bases) that may have two distinct secondary structures. The CD magnitudes for the two self-complexed forms of the polymer, while large, were no larger than we have observed for the protonated forms of other simple sequences, such as poly[d(C-T)] and poly[d(G-C)].^{5,6} There was no differential CD scattering at long wavelengths that characterizes aggregated forms of other polymers.⁴⁶ Therefore, there was no reason to suspect that the spectra we measured were of two aggregated forms of the same secondary structure of poly[d(A-C)].

Acid Transition of Poly[d(A-T)·d(A-T)]

UV absorption and CD spectral data indicated that poly[d(A-T)·d(A-T)] underwent one acid-induced transition that occurred over a narrow range of pH (4.8 to 4.2). The transition was characterized by decreases in the negative CD bands at 262, 247, and 225nm, and an increase in the positive CD at 272nm (Figure 7). These changes were all similar to those found upon heat-denaturation of the duplex polymer to the single strands.^{36,45} The greater than 50% hyperchromicity at 260nm observed for the polymer at pH 3 with respect to the polymer at neutral pH was even more convincing evidence that the polymer denatured upon lowering the pH at 20°C. (The protonation of adenine results in only minor changes in the absorption of dAMP.³⁷)

Of the four duplex polymers we have studied with dinucleotide repeats, poly[d(A-T)·d(A-T)] was the only one which did not show evidence for a protonated secondary conformation. This is interesting, since this sequence has the potential for forming a loop-out structure similar to that formed by poly[d(C-T)] at low pH.^{1,3,4,6} For poly[d(A-T)], the helical core would be made up of $A \cdot A^+$ base-pairs (similar to the poly[d(A)·d(A⁺)] self-complex²⁶), with the intervening thymidyl residues looped out. To determine if such protonated forms of poly[d(A-T)·d(A-T)] might form at a higher ionic strength, we added NaCl (to 0.5M) to a sample of the denatured polymer at 0.01M Na⁺ at pH 3. The addition of NaCl resulted in the polymer being hypochromic in the range of 210 to 300nm. (The absorbance at 260 of the polymer decreased to about the same value as that of the polymer at neutral pH.) The CD spectrum of the polymer at 0.5M Na⁺ (pH 3.1) was also similar to that of the polymer at neutral pH (data not shown). Thus, the form of poly[d(A-T)·d(A-T)] found at neutral pH, was the stable conformation at low pH and the higher ionic strength.

CONCLUSION

Poly[d(A-C)·d(G-T)] underwent two reversible transitions as the pH was lowered, one ending at pH 3.9 and the other at pH 3.4 (at 0.01M Na⁺, 20°C). Our spectral data indicated that the first transition resulted in a modified secondary conformation of the duplex polymer, while the second transition consisted of the denaturation of the protonated duplex to the single strands. Since cytosine appears to be the primary site of protonation of double-

stranded DNA, one possibility for the modified duplex at the end of the first transition may be one with protonated G·C⁺ base-pairs, alternating with A·T base-pairs between antiparallel strands. Although it is possible that the conformational change was due to a non-specific effect of protons and that the duplex structure still contained normal Watson·Crick base pairs, this seems unlikely since poly[d(A-T)·d(A-T)] did not change its structure prior to acid-denaturation.

Poly[d(A-C)] formed two self-complexes at low pH, neither of which were included in the acid-induced rearrangements of poly[d(A-C)·d(G-T)]. We do not know the type of base pairing in the form at pH 5.5. The form of poly[d(A-C)] at pH 4.5 probably contained C·C⁺ base-pairs. Poly[d(G-T)] did not form any self-complex at low pH at either 0.01M or 0.1M Na⁺.

On the basis of NMR data obtained from protonated calf-thymus DNA, Banville *et al.*⁴⁰ proposed that disruption of the duplex was the only result of protonation of the bases. While this model may be true for random sequences and certain repeating sequences of DNA such as poly[d(A-T)·d(A-T)], our spectral studies with simple, repeating, cytosine-containing, double-stranded polymers such as poly[d(G-C)·d(G-C)] and poly[d(A-G)·d(C-T)], show that these sequences form interesting protonated conformations prior to denaturation, with denaturation occurring only at very low pH.^{5,6} The data presented here for poly[d(A-C)·d(G-T)] also show that denaturation is not the only result of protonation of this polymer.

Our studies with simple sequences clearly show that it is the sequence, and not just the base composition, the types of base-pairs, or the alternation of purines and pyrimidines, that plays a crucial role in determining the conformational variability of the polymer at low pH. For example, the polypurine·polypyrimidine sequence poly[d(A-G)·d(C-T)] showed six different structural rearrangements that included a protonated triplex and protonated self-complexes of the individual strands. However, poly[d(A-C)·d(G-T)] which, like poly[d(A-G)·d(C-T)] has A·T base-pairs alternating with G·C base-pairs, but with alternating purines and pyrimidines on each strand, showed much less conformational variability at low pH, forming only one protonated structure before denaturing to the single strands at low pH. Poly[d(G-C)·d(G-C)], poly[d(A-C)·d(G-T)], and poly[d(A-T)·d(A-T)] are all alternating purine-pyrimidine sequences, but poly[d(G-C)·d(G-C)] forms two protonated secondary structures,⁵ while, as shown in this work, poly[d(A-C)·d(G-T)] formed only one protonated secondary structure, and poly[d(A-T)·d(A-T)] did not form any distinct protonated conformation upon lowering of the pH.

ACKNOWLEDGEMENTS

This work was performed by V.P.A. in partial fulfillment of the requirements for the Ph.D. degree in the Molecular and Cell Biology Program at the University of Texas at Dallas. We benefited from helpful discussions with Dr. E. Edwards, and we thank Marilyn Vaughan for her assistance with the figures. This work was supported by NIH Research Grant GM19060 and by Grant AT-503 from the Robert A. Welch Foundation. Work at the Los Alamos National Laboratory was supported in part by the U.S. Department of Energy.

REFERENCES

1. Gray, D.M., Vaughan, M.R., Ratliff, R.L., and Hayes, F.N. (1980) Nucl. Acids Res. 8, 3695–3707.

2. Gray, D.M., Cui, T., and Ratliff, R.L. (1984) Nucl. Acids Res. 12, 7565–7580.
3. Gray, D.M., Ratliff, R.L., Antao, V.P., and Gray, C.W. (1988) In Sarma, M.H. and Sarma R.H. (eds), *Structure and Expression*, Volume 2, *DNA and Its Drug Complexes, Proceedings of the Fifth Conversation in Biomolecular Stereodynamics*, Adenine Press, Guilderland, NY, pp. 147–166.
4. Brown, D.M., Gray, D.M., Patrick, M.H., and Ratliff, R.L. (1985) Biochemistry 24, 1676–1683.
5. Antao, V.P., Gray, C.W., Gray, D.M., and Ratliff, R.L. (1986) Nucl. Acids Res. 14, 10091–10112.
6. Antao, V.P., Gray, D.M., and Ratliff, R.L. (1988) Nucl. Acids Res. 16, 719–738.
7. Edwards, E.L., Ratliff, R.L., and Gray, D.M. (1988) Biochemistry 27, 5166–5174.
8. Edwards, E.L., Patrick, M.H., Ratliff, R.L., and Gray, D.M. (1990) Biochemistry 29, 828–836.
9. Gray, D.M. and Bollum, F.J. (1974) Biopolymers 13, 2087–2102.
10. Marck, C., Thiele, D., Schneider, C., and Guschlbauer, W. (1978) Nucl. Acids Res. 5, 1979–1996.
11. Hamada, H. and Kakunaga, T. (1982) Nature 298, 396–398.
12. Hamada, H., Petrino, M.G., and Kakunaga, T. (1982) Proc. Natl. Acad. Sci. USA 79, 6465–6469.
13. Pardue, M.L., Lowenhaupt, K., Rich, A., and Nordheim, A. (1987) EMBO J. 6, 1781–1789.
14. Braaten, D.C., Thomas, J.R., Little, R.D., Dickson, K.R., Goldberg, I., Schlessinger, D., Ciccocioppa, A., and D'Urso, M. (1988) Nucl. Acids Res. 16, 865–881.
15. Vorlickova, M., Kypr, J., Stokrova, S., and Sponar, J. (1982) Nucl. Acids Res. 10, 1071–1080.
16. Zimmer, C., Tymen, S., Marck, C., and Guschlbauer, W. (1982) Nucl. Acids Res. 10, 1081–1091.
17. Wells, R.D., Miglietta, J.J., Klysik, J., Larson, J.E., Sturdivant, S.M., and Zacharias, W. (1982) J. Biol. Chem. 257, 10166–10171.
18. Haniford, D.B. and Pulleyblank, D.E. (1983) Nature 302, 632–634.
19. Nordheim, A. and Rich, A. (1983) Proc. Natl. Acad. Sci. USA 80, 1821–1825.
20. McIntosh, L.P., Grieger, I., Eckstein, F., Zarlign, D.A., van de Sande, J.H., and Jovin, T.M. (1983) Nature 304, 83–86.
21. Woisard, A. and Fazakerley, G.V. (1986) Biochemistry 25, 2672–2676.
22. Ridoux, J.-P., Liqueur, J., and Taillandier, E. (1987) Nucl. Acids Res. 15, 5813–5822.
23. Riazance, J.H., Johnson, W.C., Jr., McIntosh, L.P., and Jovin, T.M. (1987) Nucl. Acids Res. 15, 7627–7636.
24. Thiele, D., Saroucci, M.-T., Guschlbauer, W., Lezius, A., and Marck, C. (1973) Mol. Biol. Rep. 1, 155–160.
25. Vorlickova, M. and Kypr, J. (1985) J. Biomol. Struct. Dyn. 3, 67–83.
26. Adler, A.J., Grossman, L., and Fasman, G.D. (1969) Biochemistry 8, 3846–3859.
27. Hunter, W.N., Brown, T., Anand, N.N., and Kennard, O. (1986) Nature 320, 552–555.
28. Sarma, M.H., Gupta, G., Sarma, R.H., Bald, R., Engelke, U., Oei, S.L., Gessner, R., and Erdmann, V.A. (1987) Biochemistry 26, 7707–7715.
29. Kalnik, M.W., Kouchakdjian, M., Li, B.F.L., Swann, P.F., and Patel, D.J. (1988) Biochemistry 27, 100–108.
30. Puglisi, J.D., Wyatt, J.R., and Tinoco, I. Jr. (1990) Biochemistry 29, 4215–4226.
31. Wells, R.D. and Blair, J.E. (1967) J. Mol. Biol. 27, 273–288.
32. Wells, R.D., Larson, J.E., Grant, R.C., Shortle, B.E., and Cantor, C.R. (1970) J. Mol. Biol. 54, 465–497.
33. Allen, F.S., Gray, D.M., and Robert, G.P., and Tinoco, I., Jr. (1972) Biopolymers 11, 853–879.
34. Gray, D.M. and Ratliff, R.L. (1977) Biopolymers 16, 1331–1342.
35. Gray, D.M., Liu, J.-J., Ratliff, R.L., and Allen, F.S. (1981) Biopolymers 20, 1337–1382.
36. Sang, B.-C. and Gray, D.M. (1989) Biochemistry, 28, 9502–9507.
37. Antao, V.P. (1988) Ph.D. thesis, The University of Texas at Dallas.
38. Fasman, G.D. (ed) (1975) In *Handbook of Biochemistry and Molecular Biology*, Vol. I, *Nucleic Acids*, Chem. Rubber Co., Cleveland, OH, pp. 76–206.
39. O'Connor, T., Mansy, S., Bina, M., McMillin, D.R., Bruck, M.A., and Tobia, R.S. (1982) Biophys. Chem. 15, 53–64.
40. Banville, D.L., Marzilli, L.G., and Wilson, W.D. (1986) Biochemistry 25, 7393–7401.
41. Htun, H. and Dahlberg, J.E. (1988) Science 241, 1791–1796.

- 42. Johnston, B.H. (1988) *Science* *241*, 1800–1804.
- 43. Pulleyblank, D.E., Haniford, D.B., and Morgan, A.R. (1985) *Cell* *42*, 271–280.
- 44. Patel, D.J., Kozlowski, S.A., Ikuta, S., and Itakura, K. (1984) *Biochemistry* *23*, 3218–3226.
- 45. Greve, J., Maestre, M.F., and Levin, A. (1977) *Biopolymers* *16*, 1489–1504.
- 46. Shin, Y.A. and Eichhorn, G.L. (1984) *Biopolymers* *23*, 325–335.