Circular dichroism of two conformations of poly[d(G-C)] induced by low pH

Vincent P. Antao, Carla W. Gray, Donald M. Gray* and Robert L. Ratliff¹

Program in Molecular Biology, The University of Texas at Dallas, Box 830688, Richardson, TX 75083-0688 and ¹Genetics Group, Life Sciences Division, Los Alamos National Laboratory, Box 1663, Los Alamos, NM 78545, USA

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ABSTRACT

Circular dichroism (CD) and UV absorption data showed that poly[d(G-C)] (at 0.09M NaCl, 0.01M Na⁺(phosphate), 20°C) underwent two conformational transitions upon lowering of the pH by the addition of HCl. The first transition was complete at about pH 3.0. The second transition was complete upon lowering the pH to 2.6 or upon raising the temperature, at pH 3.0, to about 40°C. There was no indication of denaturation during either transition. The CD spectrum for the second acid conformation had large CD bands including a positive one at 288nm, a characteristic associated with C•C⁺ base-pairs. Electron microscopy showed no significant formation of condensed supra-molecular aggregates corresponding to the first or second acid forms of poly-[d(G-C)]. On the basis of spectral data, electron microscopy, and proton-uptake measurements, we propose models for the secondary structures that poly[d(G-C)] adopts in its two acid conformations.

INTRODUCTION

The protonated self-complexes formed by poly[r(C)]^{1,2} and poly[d(C)]^{3,4} in solution are double-stranded helices stabilized by C•C⁺ base-pairs. These polymers require 0.5 proton per cytosine for maximum helix formation. Poly-[d(T-C)] also forms a protonated self-complex with the concomitant uptake of 0.25 proton per base or 0.5 proton per cytosine.^{5,6} CD spectral changes that occur upon protonation of poly[d(T-C)], such as the appearance of a large positive 288nm band, are similar to those seen with poly[d(C)] and poly[r(C)] upon formation of their acid self-complexes. A calculated CD spectrum, the average of spectra of the poly[d(C)] acid self-complex and dTMP, was found to match the measured CD spectrum of the poly[d(T-C)] acid self-complex.⁵ It was therefore proposed that the poly[d(T-C)] self-complex consists of a helical core of C•C⁺ base-pairs with the intervening thymidyl residues looped out.⁵ This loop-out model was further validated by a photochemical study which showed that cytosine<thymine dimerization decreases and cytosine<cytosine dimerization increases in the poly[d(T-C)] self-complex, relative to the dimerization in single-stranded poly[d(T-C)].⁶ From CD evidence,

the possibility has been raised that poly[d(G-C)] and poly[d(A-C)] might also adopt loop-out conformations under acid conditions.⁵

The type of cytosine base-pairing that occurs in the acid self-complexes of poly[d(C)] and poly[d(T-C)] is not known. However, hemi-protonated C·C⁺ base-pairs of the type shown in Figure 1(A) do exist in the crystal structure of cytosine-5-acetic acid.⁷ This type of base-pairing requires that the strands be parallel or that the N-glycosyl bonds be syn in one strand and anti in the other. The same kind of hemi-protonated C·C⁺ base-pairing has been seen between parallel strands in CpA co-crystallized with proflavine⁸ and also in the ammonium salt of d(CpG) crystallized from aqueous acetone near pH 4.⁹ CD studies have shown that C·C⁺ base-pairs can co-exist with Watson-Crick base-pairs between antiparallel strands of an oligodeoxynucleotide helix in solution.¹⁰ Hence, C·C⁺ base-pairs can apparently exist between either parallel or antiparallel strands of a nucleic acid.

For polymers containing G·C base-pairs, acidification can introduce a number of possible rearrangements of base-pairing. Poly[d(G)·d(C)] disproportionates to give poly[d(G)] and poly[d(C)] acid self-complexes.^{4,11} Various models have been proposed to explain the increased sensitivity of poly[d(T-C)·d(G-A)] to single-strand specific nucleases upon acidification. Pulleyblank *et al.*¹² suggest that this might be due to the formation of a protonated ^{syn}G·C⁺ base-pair alternating with A·T base-pairs. On the other hand, Lee *et al.*^{13,14} propose that poly[d(T-C)·d(G-A)] and poly[d(T-me⁵C)·d(G-A)] dismutate upon protonation to give a triplex and a poly[d(G-A)] strand which may itself form a self-complex. The C·G·C⁺ base-pairing proposed for the triplex requires the protonation of one-half of the cytosines. Interestingly, the dismutation of the methylated polymer occurs at neutral pH.¹⁴ The crystal structure of the d(GCGTACGC)-triostin A complex showed the existence of two protonated ^{syn}G·C⁺ base-pairs at pH 6.5.¹⁵ Recent optical studies of poly[d(G-C)] suggest that base protonation facilitates the rate of the NaCl-induced B → Z interconversion.^{16,17} The rates for the salt-induced B → Z transition and the actinomycin D-induced reverse Z → B transition are at least an order of magnitude faster at pH 3.8 than at neutral pH.¹⁶ Robert-Nicoud *et al.*¹⁷ observed a dramatic increase in anti-Z-DNA antibody binding to *Chironomus thummi* larvae chromosomal DNA when the DNA was exposed first to low pH. This would further suggest a relationship between protonation of the bases and the Z conformation.

From CD spectra obtained upon protonation of poly[d(G-C)] (0.15M NaCl, 25°C), Thiele *et al.*¹⁸ (1973) claimed that this polymer underwent one rever-

Figure 1. (A) The type of $G \cdot C^+$ base-pair found in crystals.^{7,8,9}
 (B) The type of $G \cdot G$ base-pair found in the ammonium salt of d(CpG) crystallized from aqueous acetone near neutral pH.⁹

sible acid-induced transition when it was titrated down to pH 2.4. Narasimhan and Bryan¹⁹ (1975) obtained CD spectra of protonated poly[d(G-C)] at a lower ionic strength (0.02M NaCl) and suggested that the CD spectral changes they observed were similar to those seen upon protonation of natural DNA, but unlike those seen when poly[d(G)·d(C)] was protonated. We have performed detailed acid titrations of poly[d(G-C)] at both 0.1M and 0.01M Na^+ . Our data showed that there are two reversible acid transitions. Acid titrations of poly[d(G-me⁵C)] and poly[r(G-C)], on the other hand, resulted in only one transition prior to denaturation.

MATERIALS AND METHODS

Poly[d(G-C)], poly[d(G-me⁵C)], and poly[d(G)] were purchased from Pharmacia P-L Biochemicals. Poly[d(C)] was purchased from Miles Laboratories. Poly[r(G-C)] was synthesized by methods used previously for other RNAs.²⁰ 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 neutral pH. Poly[r(G-C)] was finally dialyzed against 0.001M Na⁺(phosphate), pH 7.0. The extinction coefficients (ϵ_{260}) used for double-stranded poly[d(G-C)·d(G-C)], double-stranded poly[d(G-me⁵C)·d(G-me⁵C)], single-stranded poly[d(G)], the acid self-complex of poly[d(C)], and double-stranded poly[r(G-C)·r(G-C)] were, respectively, 6,960,²¹ 6,890,²² 8,990,²³ 5,300,²⁴ and 6,560^{20,25} liter·mol⁻¹·cm⁻¹. The oligomers dCMP, dGMP, d(CpC), d(GpG), and [d(G)]₈ were all purchased from Pharmacia P-L Biochemicals and the ϵ_{260} values used were, respectively, 7,400, 11,500, 7,300, 10,800, and 10,275 liter·mol⁻¹·cm⁻¹. The ϵ_{260} for [d(G)]₈ was calculated using a first-neighbor equation: $\epsilon\{[d(G)]_8\} = (7/4)\epsilon\{[d(GpG)]\} - (3/4)\epsilon\{dGMP\}$. The other ϵ_{260} values were taken from Cantor *et al.*²⁶ The mononucleotides, dinucleotides and the oligonucleotide were dissolved in 0.01M Na⁺(phosphate) at neutral pH.

UV absorption spectra were measured with a Cary-Varian Model 118 spectrophotometer. Circular dichroism measurements were made using a Cary Model 61 circular dichrometer, calibrated as described previously.⁵ CD values are given as $\epsilon_L - \epsilon_R$ in units of liter·mol⁻¹·cm⁻¹. Sample temperatures were controlled thermoelectrically to within $\pm 0.5^\circ\text{C}$. Digitized CD spectra collected at 1-nm intervals were smoothed by a sliding 13-point quadratic-cubic function.²⁷ Concentrations were determined from absorbance measurements and the extinction coefficient values mentioned above.

Solid NaCl was added to the samples to obtain the desired Na⁺ concentration. Unless otherwise specified, the titration of samples was carried out at 0.1M Na⁺ (0.09M NaCl plus 0.01M Na⁺ from sodium phosphate buffer) and 20°C. The pH was lowered by the addition of small aliquots of dilute HCl and brought back to neutral pH with small aliquots of NaOH solutions. Spectra were corrected for dilution by HCl and NaOH, although the dilution factors were never more than 5% and 2%, respectively. The pH of the samples was measured directly, using a Beckman Century SS-1 pH meter and a Beckman 5mm Futura Combination electrode. The reported pH values are accurate to within ± 0.05 pH unit.

To determine the extent of depurination during the acid titrations,

Figure 2. CD spectra of poly[d(G-C)] at 0.1M Na⁺, 20°C: at pH 6.9 (---); at pH 4.5 (o o o o); at pH 4.0 (* * * *); at pH 3.7 (+ + + +); at pH 3.4 (x x x x); at pH 3.3 (● ● ● ●); at pH 3.0 (———). Inset: the acid titration of poly[d(G-C)] as monitored by the CD at 288nm (▲) and 251nm (●); back titration at 288nm (△) and 251nm (○).

Figure 3. CD spectra of poly[d(G-C)] at 0.1M Na⁺, 20°C: at pH 3.0 (o o o o); at pH 2.8 (+ + + +); at pH 2.6 (x x x x); denatured form of poly[d(G-C)] at 0.01M Na⁺, 65°C at pH 3.4 (—); average of the CD spectra of poly[d(C)·d(C⁺)] and dGMP (- - -).

samples of poly[d(G-C)] were subjected to the extremes of pH and temperature encountered in the experiments. The samples were then subjected to perchloric acid precipitation at 0°C and quickly passed through a 0.45 μ m Millipore filter. The extent of depurination was measured as the non-precipitable material in the filtrate. A standard for a completely depurinated sample was prepared by heating duplicate samples of poly[d(G-C)] at pH 2.3 for one hour at 55°C and then for one hour at 90°C. The extent of depurination in such a standard was taken as 100%. When compared with this standard, the depurination of samples equivalent to those used during the most extreme conditions in the experiments was less than 2.5%.

For the proton-uptake measurements, poly[d(G-C)] was finally dialyzed into 0.1M Na⁺ (0.09M NaCl + 0.01M Na⁺(phosphate)), pH 7, after being dialyzed as described above. Data from each of four experiments were obtained as follows: three 2.5ml aliquots of the dialysis buffer were titrated at room temperature (24°C) to below pH 2.5 by the addition of 0.1N or 0.02N HCl, with constant stirring of the solution. HCl was added to give pH increments of 0.05 to 0.2. The pH was measured as described above. The procedure was then repeated with the dialyzed sample of poly[d(G-C)], containing from 1.8 to 3.7 μ mol of the polymer per 2.5ml (0.72 to 1.48 mM). The difference in hydrogen ion concentration between the buffer and the sample, obtained from the pH values (upon addition of equal aliquots of acid), was divided by the nucleotide concentration of poly[d(G-C)] to give the number of protons taken up per nucleotide, at the measured pH of the sample.

For electron microscopy, polymers from solutions used for spectroscopy were used without dilution or were diluted 1:10 in the same phosphate buffers (0.1M Na⁺, pH 6.8, 3.4, or 3.0) as were used for spectroscopy. The polymers were adsorbed directly onto glow-discharge-activated (hydrophilic) carbons, excess buffer was removed, and the adsorbed molecules were stained with 2% uranyl acetate.²⁸ Magnifications in the Zeiss EM10CA microscope were calibrated to $\pm 1.5\%$ (combined errors) with a Fullam diffraction grating replica; lengths were measured with a Numonics 1240 digital tracer.

RESULTS

Two Acid-Induced Spectral Transitions of Poly[d(G-C)]

Circular dichroism and UV absorption data of poly[d(G-C)] at 0.1M Na⁺ indicated that two acid-induced transitions occurred as the pH was decreased. The CD changes characterizing these transitions are shown in Figures 2 and 3. As shown in the inset to Figure 2, the first transition occurred between neutral pH and pH 3.0 and was characterized by large decreases in the magni-

Figure 4. UV absorption spectra of poly[d(G-C)] at 0.1M Na⁺, 20°C: at pH 6.9 (x x x x); at pH 3.0 (o o o o); at pH 2.8 (+ + + +); at pH 2.6 (* * * *); denatured form of poly[d(G-C)] at 0.01M Na⁺, 65° at pH 3.4 (—).

tudes of CD bands at 274, 251 and 206nm. An isodichroic point at 262nm indicated that two distinct forms of the polymer were present in the pH range 7.0 to 3.7. Below pH 3.7, the CD spectra showed the appearance of a small negative band at longer wavelengths (300-310nm) and the formation of a distinct CD band at 288nm. The UV absorption data (Figure 4) corresponding to these CD spectral changes showed hypochromicity at 256nm and hyperchromicity above 275nm. The maximum hypochromicity at 256nm and the maximum magnitudes of the CD bands at 220nm and 256nm were observed at pH 3.0. Since the spectral changes throughout the pH range 7.0 to 3.0 were only slightly different above and below pH 3.7, we considered there to be only one major conformational transition over this pH range, although there were indications (such as the appearance of the small 288nm CD band) that more than two forms of the polymer may have been present between pH 3.7 and 3.0.

Our spectral data indicated that a second major acid transition occurred between pH 3.0 and pH 2.6. The CD spectral changes for this transition were characterized by the appearance of large positive and negative CD bands at 288 and 266nm, respectively (Figure 3). There was also a reversal of the sign of the CD bands at 242 and 218nm. Isodichroic points were observed at 278, 250, and 232nm, making it likely that there was only one transition in this pH range. The UV absorption spectra (Figure 4) over this small pH range showed increasing hyperchromicity between 320 and 210nm, compared with the spectrum at pH 3.0, but the UV absorbance at 256nm of the form at pH 2.6 was still less than the absorbance of the form at neutral pH.

None of the UV absorption or CD spectra obtained during either acid transition matched the UV absorption or CD spectrum of the denatured form at neutral pH (data not shown) or at low pH (Figures 3 and 4).

The second spectral transition could also be induced by raising the temperature at pH 3.0 and was complete at about 42°C (Figure 5); spectra taken in the range 42-50°C showed no significant changes. The UV absorption and CD spectra at the end of this temperature-induced second acid transition remained unchanged upon lowering the temperature to 20°C, even after several days. The structure at the end of the second transition showed evidence of melting when heated above 50°C (pH 3.0 and 0.1M Na⁺). When the polymer at pH 3.3 was heated, no changes were observed in the UV absorption or CD spectra up to 45°C (data not shown). Above 45°C, the latter sample showed spectral changes characteristic of melting. The second spectral transition also occurred for samples of poly[d(G-C)] at pH 3.0 that were held at 20°C for several hours (data not shown). The UV absorption and CD spectra of

Figure 5. CD spectra of poly[d(G-C)] at 0.1M Na⁺, pH 3.0: at 20°C (—); at 25°C (o o o o); at 30°C (+ + + +); at 35°C (x x x x); at 40°C (• • • •); at 42°C (- - - -).

the polymer at pH 3.4 and pH 3.3 did not change for at least one hour at 20°C (data not shown).

Both of the acid-induced transitions were largely reversed upon back titration with NaOH. Taking the magnitude of the 251nm CD band as an indication of how much of the polymer reverted to its starting conformation (at pH 7), we estimated that it was over 80% reversed. However, there was hysteresis in the optical properties over the pH range of the second transition (inset, Figure 2). Thus, the pKa values for the second transition differed by about 0.6 pH unit during the acid and alkali titrations.

When an acid titration was carried out with poly[d(G-C)] at 8.5μM (1/25 the concentration usually used), the CD spectra during the two transitions were qualitatively the same as seen at higher concentrations (data not shown). However, the magnitude of the spectra during the second transition was 1/3 to 1/2 that seen with the polymer at the usual concentration, indicating that the second transition was dependent on the polymer concentration.

Electron Microscopy of Poly[d(G-C)] at Low pH

Electron microscopy was used to monitor samples from three repeat pH titrations of poly[d(G-C)] in 0.1M Na⁺; micrographs of unselected fields of view (examining several thousand molecules for each set of conditions) gave uniform and consistent results throughout (data not shown). The polymer molecules at pH 6.8 and 3.4 had smooth, curvilinear contours and measured widths of about 2nm, being indistinguishable in these respects from double-stranded PM2-DNA used as a control. A number of branches occurred, presumably due to the formation of "Y" joints at junctions between concatenated polymer strands. Linear segments bounded by branches or molecular ends measured 6nm to 390nm in length. Poly[d(G-C)] heated at pH 3.0 to convert it to the second acid form showed about a twofold reduction in the mean segment length, possibly the result of strand breakage during heating. Although occasional tangled networks of molecules occurred under all conditions tested, these appeared likely to be the result of interlocking by a three-dimensional network of branches. The polymer widths were always about 2-3nm, even in the networks; there was no significant condensation nor extensive side-by-side aggregation to form thick filaments. Thus, we did not find any evidence that the acid structures of poly[d(G-C)] resembled the supramolecular condensates described by Castleman *et al.*²⁹ for the Z-form of this polymer.

Proton-Uptake Experiments with Poly[d(G-C)]

The number of protons taken up per nucleotide was determined as described in Materials and Methods. There was no detectable uptake of protons

Figure 6. Proton uptake of poly[d(G-C)] at 0.1M Na⁺, 24°C, plotted as the number of protons taken up per nucleotide as a function of pH. Data from four different experiments with different concentrations of polymer are shown: 0.72mM (○); 0.97mM (△); 1.40mM (▲); 1.48mM (●).

(—X—X—) The acid titration of poly[d(G-C)] as monitored by the 251nm CD band (from Figure 1).

down to pH 5, and then the uptake was gradual with about 0.25 and 0.5 proton per base being taken up at the ends of the first and second transitions, respectively (Figure 6). To determine if the additional protons taken up by the polymer during the second acid transition were necessary to form the second conformation, a similar concentrated sample of poly[d(G-C)] was titrated to the end of the first transition, and the second transition was induced by raising the temperature to 42°C. The temperature was then decreased to 20°C. CD spectra were recorded in a 1mm pathlength cell to confirm that the transition had occurred. The pH of the sample after heating and cooling was the same as that before heating; that is, it remained at pH 3.0 and did not increase to pH 3.2 as would be expected for this sample if an additional 0.25 proton per base had been taken up. Thus, the number of protons taken up by the polymer did not increase and remained at 0.25 proton per base. This indicated that added protons were not an obligatory part of the second acid structure and that decreasing the pH to below 3.0, like heating the polymer at pH 3.0 or allowing the polymer to stand at 20°C at pH

Figure 8. CD spectra of poly[r(G-C)] at 0.1M Na⁺, 20°C: at pH 6.8 (— — —); at pH 4.8 (o o o o); at pH 4.3 (+ + + +); at pH 3.8 (x x x x); at pH 3.4 (• • • •); at pH 2.8 (—). Inset: the acid titration of poly[r(G-C)] as monitored by the CD at 293nm (▲) and 263nm (●).

3.0, was only a means of destabilizing the first acid conformation and inducing the second acid conformation.

Effect of Salt Concentration on Poly[d(G-C)] Acid Transitions

Two acid-induced transitions were also observed in CD spectra of poly[d(G-C)] at the lower salt concentration of 0.01M Na⁺(phosphate), 20°C. However, the pKa values of the transitions were about 0.4 pH unit higher than for the polymer at 0.1M Na⁺, 20°C. Thus, the acid forms of poly[d(G-C)] were more stable at 0.1M Na⁺ than at 0.01M Na⁺. The relative instability (as a function of pH and temperature) of the second acid form at the lower salt concentration probably explains why Narasimhan and Bryan¹⁹ did not observe CD spectra representing the second acid form. UV absorption and CD spectra identified as characteristic of the protonated, denatured form of poly[d(G-C)] (by the extent of increase in UV absorbance and the low intensity of the CD bands) were obtained in 0.01M Na⁺(phosphate) at pH 2.6, 20°C, or at pH 3.4, 65°C (spectra under the latter conditions are shown in Figures 3 and 4). Titrating poly[d(G-C)] to a pH value below that at which the second acid structure formed resulted in denaturation of the polymer; however, at 0.1M Na⁺, precipitation of the polymer began to occur before denaturation was complete. At 0.01M Na⁺ the denatured polymer did not precipitate and could be titrated back with NaOH to give the two acid secondary structures and the neutral pH conformation.

Acid Titration of Poly[d(G-me⁵C)]

Upon titration of poly[d(G-me⁵C)] (at 0.1M Na⁺) down to pH 2.6, only one transition was observed (Figure 7). The CD spectrum of poly[d(G-me⁵C)] at neutral pH resembled that of poly[d(G-C)]. The CD spectra (pH 3.3 to pH 2.6) of poly[d(G-me⁵C)] toward the end of its only acid transition, qualitatively resembled the CD spectrum of poly[d(G-C)] at the end of its first transition. For example, in both cases there were five crossover points above 210nm with positive and negative CD bands at similar wavelengths. (Compare CD spectra shown as solid lines in Figures 2 and 7). When poly[d(G-me⁵C)] at lower ionic strength, 0.01M Na⁺(phosphate), was titrated to pH 2.6 at 20°C or heated to 50°C at pH 3.0, the polymer denatured. Apparently the methyl group on the C5 of cytosine did not permit the polymer to undergo the second acid transition found for poly[d(G-C)] under similar conditions.

Acid Titration of Poly[r(G-C)]

At neutral pH and 0.1M Na⁺, the poly[r(G-C)] was in the A conformation.²⁰ Upon acid titration of this polymer to pH 2.8, only one gradual transition was observed (Figure 8). CD spectral changes observed for the acid-induced transition of poly[r(G-C)] differed from those seen upon melting

this polymer at neutral pH (unpublished data; K.H. Johnson, The University of Texas at Dallas). On lowering the pH to 2.8, the UV absorption at 259nm decreased. A further decrease in pH to 2.4 resulted in denaturation of the polymer to the protonated single-stranded form as evidenced by an increase in absorption as well as by the similarity of the CD spectrum to that of the single-stranded form at neutral pH. At 0.01M Na⁺(phosphate), poly[r(G-C)] underwent the same acid transition as it did at the higher salt concentration (0.1M Na⁺), except that the pK_a of the transition increased by 0.1-0.2 pH units, resulting in denaturation of the polymer to the single-stranded form at pH 2.8 (data not shown). The CD observed for the acid conformation differed from the CD associated with the salt-induced Z-form of this polymer.³⁰ The CD of the acid form did resemble spectra associated with the Z-form of poly[d(G-C)],^{16,31,32,33} except that the spectrum of the acid form of poly[r(G-C)] at 20°C had smaller bands and a long-wavelength crossover that was shifted to shorter wavelengths by about 10nm.

DISCUSSION

The evidence presented here clearly shows that poly[d(G-C)] underwent two acid-induced transitions, each resulting in a conformation with a distinct CD spectrum. Among the three polymers studied, poly[d(G-C)] was unique in showing two conformational states at acid pH. The UV and CD spectral data indicated that neither conformation of poly[d(G-C)] was a denatured form of the polymer. Electron microscopy showed that the acid forms of poly[d(G-C)] consisted of polymers about 2-3nm in width; thus, neither conformation consisted of more than a very small number of strands. There was no evidence for aggregates or condensates in which large numbers of DNA strands were brought into close juxtaposition. Electron microscopy also showed no evidence for extensive denaturation (collapsed single DNA strands). Poly[d(G-C)] in its acid conformations was uniformly extended and had a rigidity (curvature along the helix axis) roughly comparable to that of a B-DNA double helix. The simplest possibility consistent with all of our observations is that both acid forms of poly[d(G-C)] were modified double-helices.

A proton uptake of 0.25 proton per base was required for each of the two acid conformations of poly[d(G-C)]. The two possible sites of protonation were the N3 of cytosine and the N7 of guanine. The N3 of cytosine has a higher pK_a value than does the N7 of guanine (4.3 and 4.6 for dC and 5'-dCMP compared with 2.5 and 2.9 for dG and 5'-dGMP, respectively).³⁵ The increase in absorption at long wavelengths (270-310nm) upon acid titration of poly-

[d(G-C)] (Figure 4) was consistent with cytosine being the base that was initially protonated. On the other hand, on the basis of ORD spectra of natural DNA, it has been suggested that guanines can be protonated before cytosines since the site of protonation on cytosine, N3, is involved in a Watson-Crick base-pair.³⁵ However, this leads to the unlikely consequence that the N7 of guanine (though not involved in hydrogen-bonding) would have to take up a proton at a pH much higher than its pKa. The fact that cytosine has the higher pKa has previously been invoked to suggest that it is the base that is protonated first in double-stranded natural DNA.^{36,37} A recent Raman study on the acid titration of natural DNA solutions appears to support this view.³⁸ Dismutations of poly[d(T-C)·d(G-A)] and poly[d(T-me⁵C)·d(G-A)] at pH 5 and pH 7, respectively, to form triplexes plus free polypurine strands are thought to involve the protonation of cytosine and not guanine at these relatively high pH values.^{13,14} Finally, the crystal structure of d(CpG) at pH 4 and the structure of the d(GCGTACGC)-trioestin A complex at pH 6.5 both indicate that cytosines, not guanines, are protonated.^{9,15} Since cytosine appeared to be the most likely site of protonation during the first acid transition of poly[d(G-C)], and since both acid conformations required the same degree of protonation, we concluded that protonated cytosines were very likely involved in both acid conformations.

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

The changes in both the UV absorption and CD spectra for poly[d(G-C)] showed that the first acid transition (ending at about pH 3.0) was a broad one, occurring over a range of more than two pH units. The CD changes and the proton-uptake paralleled each other throughout most of the pH range from 7 to 3.0, although there was some deviation below pH 3.7 (Figure 6). Thus, protons were taken up gradually during the course of the first structural transition. Although the CD bands decreased in magnitude during this transition, the CD spectra increased in complexity, having five crossover points above 200nm at pH 3.0 (Figure 2). The UV absorption decreased in the region of $\lambda_{\max}$ upon acid titration down to pH 3.0, indicating that there was little or no unstacking of bases or strand separation. The first transition was also independent of the concentration of the polymer, suggesting that this transition did not involve strand-rearrangements or the formation of aggregates.

On the basis of these data, it appeared that the protons were taken up by the polymer without strand separation. Protonation of the cytosines would disrupt the normal Watson-Crick G·C base-pairs, however. Three possible arrangements for protonated G·C⁺ base-pairs are the Hoogsteen, reversed Hoogsteen, and slipped Watson-Crick arrangements.¹² We were unable to distin-

guish among these on the basis of our data. It is interesting to note that formation of the Hoogsteen G·C⁺ base-pair between antiparallel strands would require the guanine to be in the *syn* conformation. Two such *syn*G·C⁺ base-pairs have been observed by Quigley *et al.*¹⁵ in the crystal structure of the d(GCGTACGC)-triestin A complex. The formation of *syn*G·C⁺ base-pairs during the first acid transition of poly[d(G-C)] might help explain the observation by Chen¹⁶ and Robert-Nicoud *et al.*¹⁷ that protonation facilitates the B → Z transition, since all the guanines are in the *syn* conformation in the Z form of poly[d(G-C)]. The possible involvement of the *syn*G conformer in the acid facilitation of this transition has previously been pointed out by Chen.¹⁶

The uptake of 0.25 proton per base, or one proton per two cytosines, indicated that only one-half of the G·C base-pairs were eventually protonated. One possibility for the arrangement of protonated G·C⁺ base-pairs relative to the Watson-Crick base-pairs is that the formation of protonated G·C⁺ base-pairs was completely random and the cytosines were not all protonated prior to the transition to the second acid form. This possibility might be supported by the complexity of the CD spectrum at pH 3.0 and the appearance of more than two components in the CD spectrum between pH 3.7 and 3.0. That is, protonation could result in four nearest-neighbor spectral contributions ((GC)⁺·(GC), (CG)⁺·(CG), (GC)⁺·(GC)⁺, and (CG)⁺·(CG)⁺) of which the contributions of adjacent protonated pairs would increase proportionately at lower pH. Another possibility, which we cannot exclude, is that the structure at the end of the first transition consisted of a dinucleotide repeat, with the protonated and unprotonated G·C base-pairs alternating with each other. That is, the formation of a protonated G·C⁺ base-pair might preclude the adjacent base-pairs from being protonated.

It should be noted that the changes in the UV absorption spectrum of poly[d(G-C)] observed during the first acid transition were similar, both qualitatively as well as quantitatively, to those observed for the B → Z transition. (The A₂₉₅/A₂₆₀ increased from 0.10 at pH 6.8 to 0.42 at pH 3.0). The A₂₉₅/A₂₆₀, or the inverse of this ratio, is now often used to monitor the B → Z transition. It remains to be determined whether or not the acid-induced and the salt-induced transitions are related. Meanwhile, caution must be exercised in using the ratio A₂₉₅/A₂₆₀, or its inverse, as a diagnostic for the B → Z transition. Similarly, the CD spectrum of the protonated RNA poly[r(G-C)] had features resembling the Z-DNA spectrum, which reinforces the conclusion by Tomasz *et al.*³⁹ that an inverted long-wavelength CD band is not sufficient to identify a left-handed Z conformation.

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

The CD spectral changes during the second acid transition were characterized by large increases in the magnitude of the positive 288nm and the negative 266nm CD bands. A dramatic increase in the long-wavelength positive CD band (280-300nm) was observed upon protonation of cytosine-rich oligomers and polymers,^{4,5,10} but not dCMP or d(CpC) (data not shown). This change is indicative of the formation of C•C⁺ base-pairs.^{4,5,6} Poly[d(G)], [d(G)]₈, d(GpG), and dCMP did not show such dramatic changes in their long-wavelength CD at neutral pH or upon titration with acid to pH 2.5 (data not shown), indicating that interaction between guanines was not largely responsible for these CD changes. The large, positive 288nm CD band seen for the second acid form was therefore indicative of the presence of hemi-protonated C•C⁺ base-pairs. The uptake of 0.25 proton per base, or one proton for every two cytosines was in agreement with the existence of C•C⁺ base-pairs. If we assume the presence of C•C⁺ base-pairs in the second low pH conformation of poly[d(G-C)], the number of types of possible duplex structures of a given handedness is limited to four. The second acid form could contain C•C⁺ base-pairs with the intervening G residues being intrahelical (presumably paired) or extrahelical, and the strands being either parallel or antiparallel.

The appearance of the second transition at pH 3.0 as a function of time, and the other means of inducing the second transition (raising the temperature to about 40°C at pH 3.0, 0.1M Na⁺ or lowering the pH from 3.0 to 2.6 at 0.1M Na⁺, 20°C), strongly suggest that the second transition required the rearrangement of the partially protonated duplex into another form. These ways of inducing the second transition, together with the concentration dependence of the second transition and hysteresis observed upon back titration (inset, Figure 2), would be consistent with a rearrangement to form a duplex with parallel strands.

A C•C⁺ base-pair stacked on a G•G base-pair has been observed in the crystal structure of d(CpG) at low pH.⁹ The type of C•C⁺ and G•G base-pairs seen in the crystal structure of d(CpG) are shown in Figures 1A and 1B, respectively. The crystal structure of d(CpG) at low pH has parallel strands.⁹ Thus, an attractive possibility for the second acid conformation of poly[d(G-C)] is one in which C•C⁺ and intrahelical G•G base-pairs stack as in the crystal structure of d(CpG).⁹ An intrahelical location for the guanines would be consistent with the general tendency of guanines to stack, as illustrated by the recent work of Aboul-ela *et al.*⁴⁰ who found that the most stable mismatches in a duplex DNA oligomer were those containing

guanine. The fact that the second acid form of poly[d(G-C)] was stable up to 50°C also suggests that the guanines were paired.

On the basis of the CD spectrum of poly[d(G-C)], 0.15M Na⁺, 25°C, pH 2.5, reported by Thiele *et al.*,¹⁸ the possibility was raised by Gray *et al.*⁵ that this polymer at low pH, like poly[d(T-C)], might form a loop-out structure having a helical core of stacked C•C⁺ base-pairs with the intervening guanines looped-out. The calculated CD spectrum for such a loop-out model is shown in Figure 3. The major CD bands (positive band at 288nm, negative band at 266nm) of the spectrum measured at the end of the second transition were similar to those in the calculated CD spectrum. However, there were significant differences in the magnitudes of these two major bands in the measured and calculated spectra, and the width of the 288nm CD band was less than that seen for the self-complexes of poly[d(C)] and poly[d(T-C)].^{4,5} The UV absorption spectrum measured for the form at the end of the second transition was of a much lesser magnitude than that calculated for a simple loop-out conformation (data not shown). For example, the ϵ_{270} for the form at the end of the second transition was about 35% less than that calculated for a simple loop-out structure. The lack of agreement between the measured spectral data and those calculated for a loop-out structure, together with the strong tendency of guanines to stack and remain intrahelical in G•G mismatches, argue against the second acid conformation being a simple loop-out structure.

Acid Transitions of Other Polymers

The data obtained upon titration of other synthetic polymers containing strictly alternating guanine and cytosine residues indicated that poly[d(G-C)] was unique in undergoing two conformational transitions (excluding denaturation) upon protonation. Poly[d(G-me⁵C)] underwent only one transition which was very similar to the first acid transition of the unmethylated polymer. It did not undergo a second acid transition as in the case of poly[d(G-C)]. Poly[d(me⁵C)] formed an acid self-complex at neutral pH (data not shown) similar to that of poly[d(C)].⁴ Therefore, it was not clear why the methyl group on the C5 of cytosine prevented the formation of the second acid structure. Protonation of bases in both poly[d(G-C)] and poly[d(G-me⁵C)] facilitates the B → Z transition.^{16,17,41} As mentioned above, this facilitation might be due to the formation of ^{syn}G•C⁺ base-pairs. If this is so, the finding that the CD spectrum of the acid conformation of poly[d(G-me⁵C)] was similar to that of the first acid conformation of poly[d(G-C)] would be consistent with the possibility that both structures involved protonated base-pairs of the ^{syn}G•C⁺ type.

Summary

Poly[d(G-C)] underwent two acid transitions, one ending at pH 3.0 and the other at pH 2.6. Both resulted in definite secondary conformations of the polymer, not denatured or aggregated forms. Spectral data, electron microscopy, and proton-uptake measurements have narrowed the possible types of secondary structure and base-pairing that existed in the acid conformations of poly[d(G-C)]. The simplest possibility is that both forms were modified double helices, and protonated cytosines were most likely involved in both conformations, the first having G·C⁺ and the second having C·C⁺ base-pairs. We propose that the form at pH 3.0 consisted of about 50% protonated G·C⁺ base-pairs, possibly of the ^{SYN}G·C⁺ type between antiparallel strands, and that the form at pH 2.6 (or at pH 3.0 after heating) most likely consisted of C·C⁺ base-pairs alternating with G·G base-pairs between parallel strands, a solution form of the crystalline structure determined by Cruse *et al.*⁹ for d(CpG) at low pH.

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*To whom correspondence should be addressed

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