Proton Magnetic Resonance Melting Studies of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp†

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ABSTRACT: Proton magnetic resonance melting curves are reported for the base and ribose 1′ nonexchangeable protons of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. In general, the double helices melt in a cooperative two-state transition. For CCGGp, CCGGAp, CCGGUp, and ACCGGUp, the 3′ ribose exhibits conformational flexibility at lower temperatures than the other ribose residues. For ACCGGp, the 5′-C ribose is the first to exhibit flexibility. The conformations of the 3′ terminal bases in CCGGAp and CCGGUp change cooperatively with the double- to single-strand transition, whereas the 5′ A of ACCGGp appears to have more conformational freedom.

In a preceding paper (Petersheim & Turner, 1983a), we reported the thermodynamics for the single-strand to double-helix transitions of CCGG, CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. The thermodynamics were derived by assuming the transitions are two-state and interpreted by assuming the conformations of the CCGG cores are similar. Nuclear magnetic resonance (NMR)[1] provides a method for partially testing these assumptions. In particular, measurements of chemical shift and line width vs. temperature can be used to follow the melting behavior of each nucleotide (Borer et al., 1975; Cross & Crothers, 1971; Arter et al., 1974). Additionally, measurements of the ribose 1′–2′ coupling constants ($J_{1'2'}$) provide information on the conformation of each ribose (Jardetzky, 1960; Lee et al., 1976; Altona & Sundaralingam, 1973; Evans & Sarma, 1974; Dhingra & Sarma, 1979). In this paper, we report NMR melting curves for the base and ribose 1′ nonexchangeable protons of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. Data are also given for CCGG at 7 °C. In general, these results support the two-state and conformational assumptions used for the thermodynamics. However, subtle differential behavior with temperature is observed for some of the protons, especially with ACCGGp.

Experimental Procedures

Samples. NMR samples were prepared by first dissolving the material in 0.4–0.5 mL of H_2O containing 1.0 M NaCl, 1 mM EDTA, and 10 mM cacodylate, pH 7.0 ± 0.1, lyophilizing, and redissolving in 99.8% D_2O (Bio-Rad). The internal reference, TSP (Wilmad), was added to provide a final concentration of 1 mM. Lyophilization and dissolution in D_2O were repeated twice more.

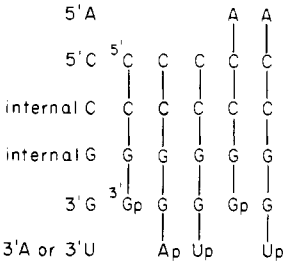
Concentrations were measured by absorbance at 280 nm with the extinction coefficients reported previously (Petersheim & Turner, 1983a). It was assumed D_2O does not affect the extinction coefficients.

NMR Experiments. NMR experiments were performed on a Bruker WH-400-MHz Fourier transform instrument with an Aspect-2000 computer. A hundred to a thousand FID accumulations were required, depending on oligomer concentration and line broadening. Temperature was controlled with a Bruker VT-1000 variable temperature unit.

Results

Plots of chemical shift vs. temperature for the base and ribose 1′ nonexchangeable protons of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp are shown in Figures 1–5. The chemical shifts at high and low temperature are listed in Tables I and II, respectively. Also listed in Table II are the chemical shifts measured for CCGG at 7 °C.

Resonance assignments for CCGGAp, ACCGGp, and ACCGGUp were made with the aid of the nuclear Overhauser effect (NOE) (Petersheim & Turner, 1983b). For CCGGp and CCGGUp, assignments were made by comparison with CCGGAp, ACCGGp, and ACCGGUp and with predicted chemical shifts (Hader et al., 1982). In describing the resonance assignments, we will refer to the residues of the oligomers as



In the three oligomers assigned by NOE, the internal G8 resonance occurs farther downfield than the 3′G8 resonance. The G8 resonances of CCGGp and CCGGUp were assigned accordingly. At high temperatures, the 5′C6 in the NOE assigned oligomers is always farther downfield than the internal C6. The 5′C6 resonances of CCGGp and CCGGUp were assigned accordingly. The low-temperature behavior of the C6 resonances for CCGGp and CCGGUp follows that of CCGGAp; i.e., the 5′C6 resonance is farther downfield than the internal C6 resonance. It was assumed that the C5 resonances for CCGGp and CCGGUp also follow their behavior in CCGGAp. Thus the resonance furthest downfield at low

† From the Department of Chemistry, University of Rochester, Rochester, New York 14627. *Received June 24, 1982.* This work was supported by National Institutes of Health Grant GM22939. D.H.T. is an Alfred P. Sloan Fellow. M.P. was a National Institutes of Health Predoctoral Trainee (Grant 5T32 GM 07230).

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[1] Abbreviations: NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate; FID, free induction decay; ppm, parts per million; NOE, nuclear Overhauser effect; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-0269$01.50/0

Table I: Observed and Calculated Chemical Shifts for Nonexchangeable Protons at High Temperature

	CCGGp (73 ± 1 °C)		CCGGAp (74 ± 1 °C)		ACCGGp (72 ± 1 °C)		CCGGUp (68 ± 1 °C)		ACCGGUp (84 ± 1 °C)	
proton[a]	calcd[b]	obsd[c]	calcd[b]	obsd[c]	calcd[b]	obsd[c]	calcd[b]	obsd[c]	calcd[b]	obsd[c]
5′A8					8.323	8.299			8.323	8.298
A2					8.204	8.205			8.204	8.230
A1′					6.059	6.049			6.059	6.052
C6	7.762	7.764	7.762	7.757	7.751	7.724	7.762	7.766	7.751	7.728
C5	5.986	6.002	5.986	5.982	5.870	5.871	5.986	5.997	5.870	
C1′	5.865	5.880	5.865	5.848	5.846	5.848	5.865	5.876	5.846	
C6	7.751	7.732	7.751	7.738	7.710	7.700	7.751	7.734	7.710	7.704
C5	5.948	5.984	5.948	5.982	5.901	5.943	5.948	5.973	5.901	
C1′	5.824	5.849	5.824	5.848	5.776	5.802	5.824	5.827	5.776	
G8	7.949	7.932	7.888	7.914	7.949	7.925	7.951	7.929	7.951	7.904
G1′	5.841	5.782	5.789	5.760	5.841	5.848	5.853	5.787	5.853	
G8	7.978	7.998	7.883	7.893	7.978	7.970	7.981	7.973	7.981	7.970
G1′	5.909	5.900	5.796	5.732	5.909	5.878	5.909	5.875	5.909	
U6							7.780	7.788	7.780	7.780
U5							5.759	5.827	5.759	5.866
U1′							5.887	5.945	5.887	5.978
A8			8.300	8.372						
A2			8.144	8.215						
3′A1′			6.065	6.104						

[a] Resonances ordered from 5′ terminus (top) to 3′ terminus (bottom). [b] Calculated from parameters determined by Hader et al. (1982); ppm from DSS; 1.0 M NaCl, 100% D_2O, 70 ± 1 °C. [c] ppm from TSP; 1.0 M NaCl, 99.8% D_2O.

Table II: Chemical Shifts (ppm) for Nonexchangeable Protons at Low Temperature[a]

proton[b]	CCGG	CCGGp[c]	CCGGAp	ACCGGp	CCGGUp	ACCGGUp
5′A8				8.327		8.500
A2				8.045		8.086
A1′				5.816		6.004
C6	7.954	8.106	8.199	7.714	8.214	7.798
C5	5.950	5.943	6.088	5.462	6.125	5.338
C1′	5.225	5.640	5.666	5.198	5.664	5.526
C6	7.965	8.003	7.923	7.887	8.020	7.865
C5	5.569	5.616	5.574	5.529	5.649	5.598
C1′	5.615	5.561	5.561	5.589	5.649	5.504
G8	7.519	7.561	7.542	7.486	7.562	7.538
G1′	5.754	5.772	5.737	5.737	5.796	5.771
G8	7.289	7.384	7.196	7.278	7.195	7.218
G1′	5.838	5.887	5.723	5.816	5.815	5.783
U6					7.559	7.643
U5					5.324	5.223
U1′					5.751	5.861
A8			7.817			
A2			7.927			
3′A1′			6.043			
temp (°C)	6.6	−3.5	8.5	2.5	7.7	6.6
concn (mM)	(1)	0.80	1.1	0.77	0.57	0.41

[a] Referenced to TSP; 1.0 M NaCl, 10 mM cacodylate, 1.0 mM EDTA, pH 7.0, 99.8% D_2O. [b] Resonances ordered from 5′ terminus (top) to 3′ terminus (bottom). [c] These data are not included in Figure 1. The line widths at this temperature are not included in Figure 6; they are 8 Hz for 3G8 and 9.2 Hz for 4G8.

temperature is assigned to the 5′C5 proton. The U6 and U5 protons of CCGGUp were assigned by their coupling constants. All the pyrimidine resonance assignments were also corroborated by decoupling experiments.

The ribose 1′ assignments for CCGGp and CCGGUp are somewhat less certain. For ACCGGUp at high temperature, the 3′U1′ resonance occurs farther downfield than the G1′ and C1′ resonances. The 3′U1′ of CCGGUp is assigned accordingly. For the three oligomers assigned by NOE, the chemical shifts of the G1′ protons change very little with temperature. This was assumed to also be true for CCGGp and CCGGUp. For ACCGGp at low temperature, the 3′G1′ resonance occurs farther downfield than the internal G1′ resonance. This was assumed to hold for CCGGp. For CCGGUp, the G1′ resonances were assigned with the predictions of Hader et al. (1982). For CCGGAp at low temperature, the internal C1′ resonance occurs furthest upfield, and the C1′ assignments in CCGGp and CCGGUp were made accordingly.

The spectrum of CCGG at 7 °C was assigned by comparison with that of CCGGp. The only unusual resonance is the ribose 1′ at 5.2 ppm. In all the other oligomers, the only ribose 1′ resonance that far upfield is due to the 5′C ribose in ACCGGp. The resonance at 5.2 ppm in CCGG was assigned to the 5′-terminal 1C1′ proton. The unusual chemical shift may be due to the absence of terminal phosphates in CCGG or to the possible presence of end-to-end aggregation. The chemical shifts for CCGG listed in Table II are very different from those reported by Arter et al. (1974). This is probably due to aggregation effects that should be more important at the 13.1 mM concentration used by Arter et al.

In Table I, the chemical shifts observed at high temperature are compared with those predicted by Hader et al. (1982). The predicted values are referenced to DSS while the measured values are referenced to TSP. The temperature dependence

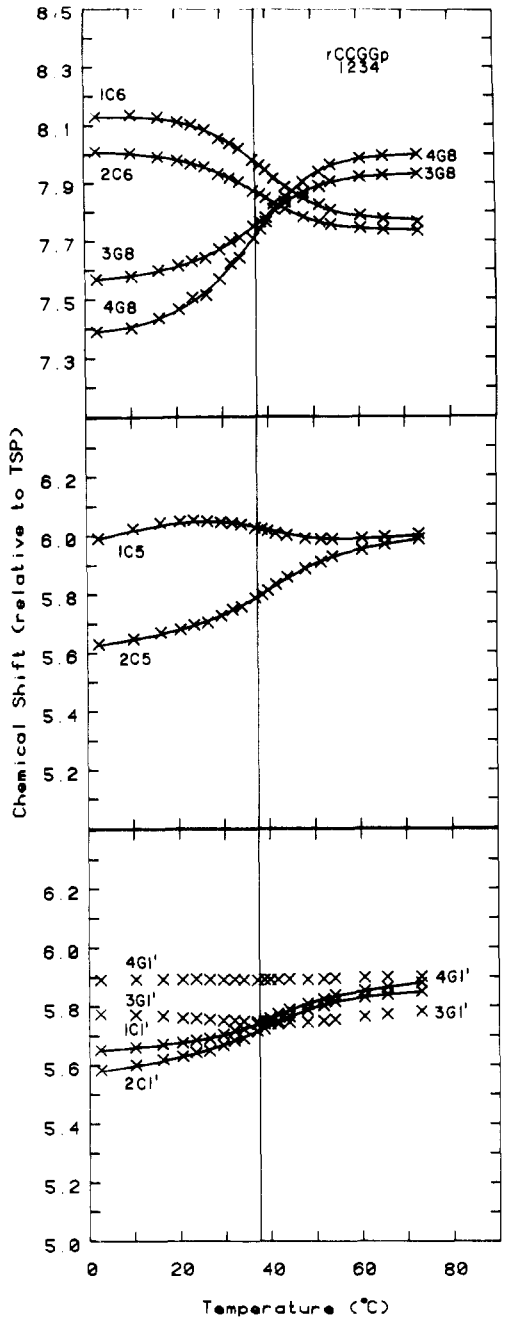

FIGURE 1: Chemical shift vs. temperature for nonexchangeable base and 1′ protons of 0.8 mM CCGGp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Solid curves are fits of the data to eq 1. The vertical line is the average of the NMR T_m's.

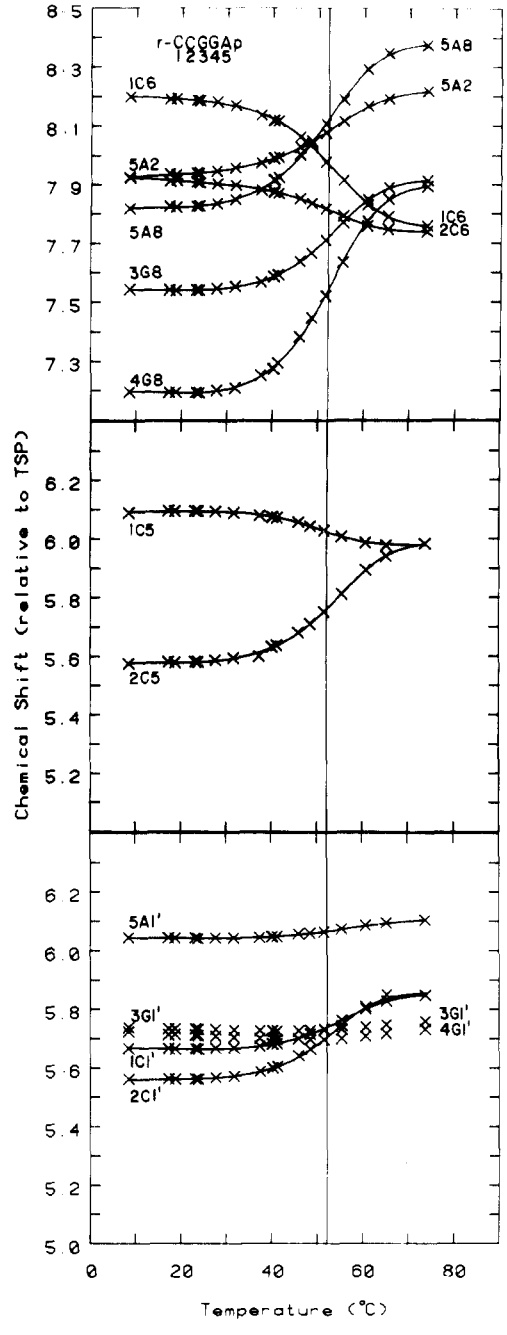

FIGURE 2: Chemical shift vs. temperature for nonexchangeable base and 1′ protons of 1.1 mM CCGGAp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Solid curves are fits of the data to eq 1. The vertical line is the average of the NMR T_m's.

of these two references are identical (Cross & Schleich, 1977), and their reported chemical shifts are within 0.015 ppm (Tiers & Kowalsky, 1960; Gordon & Ford, 1972). For the oligomer base protons, the average absolute magnitude of the difference between measured and predicted chemical shifts is 0.024 ppm, and the relative ordering of the resonances is predicted exactly. For the ribose 1′ protons, the average absolute deviation is 0.030 ppm, but the relative ordering often is not consistent with our assignments. In general, however, it appears the method proposed by Hader et al. (1982) will be very useful for assigning oligonucleotide spectra.

If the observed chemical shifts represent population-weighted averages of the shifts for the double-helix and single-strand states, then the chemical shift vs. temperature curves can be analyzed much like optical melting curves (Petersheim & Turner, 1983a). The solid lines in Figures 1–5 are fits of the data to the following function:

$$\delta(T) = (a_1T + b_1)2c_D/c_T + (a_2T + b_2)c_S/c_T \quad (1)$$

$$c_S = \frac{-1 + (1 + 8c_TK)^{1/2}}{4K} \quad (2)$$

$$K = \exp[-\Delta H/(RT) + \Delta S/R] \quad (3)$$

Here, $\delta(T)$ is the chemical shift observed at temperature T; a_1 and a_2 are slopes of the chemical shift vs. temperature for a given proton in the double- and single-strand states, respectively; b_1 and b_2 are the chemical shifts of the proton at 0 K in the double- and single-strand states, respectively; c_D and c_S are the double- and single-strand concentrations, re-

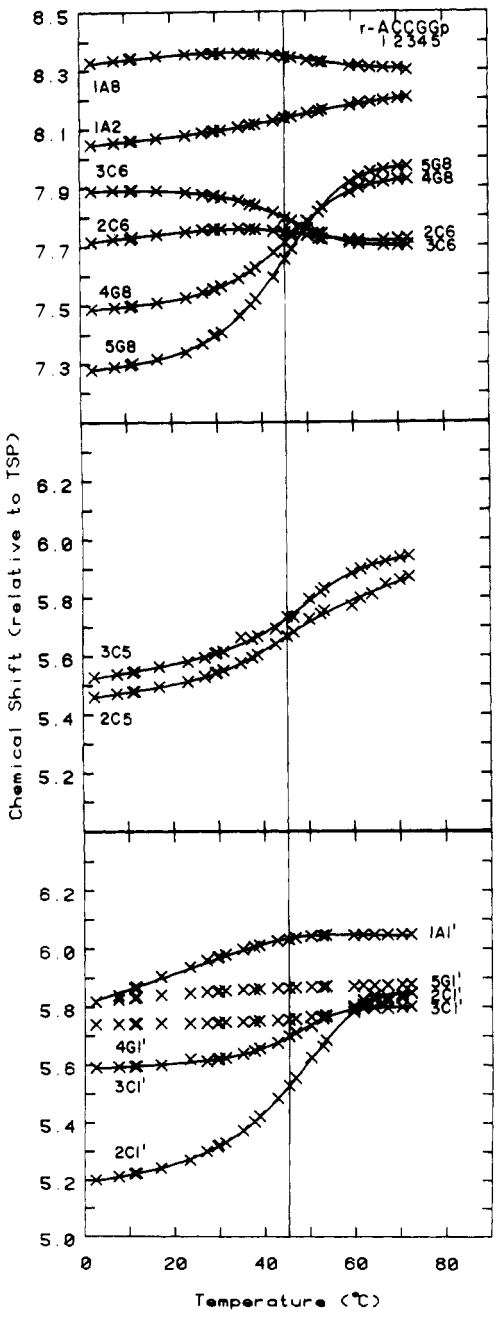

FIGURE 3: Chemical shift vs. temperature for nonexchangeable base and 1′ protons of 0.77 mM ACCGGp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Solid curves are fits of the data to eq 1. The vertical line is the average of the NMR T_m's.

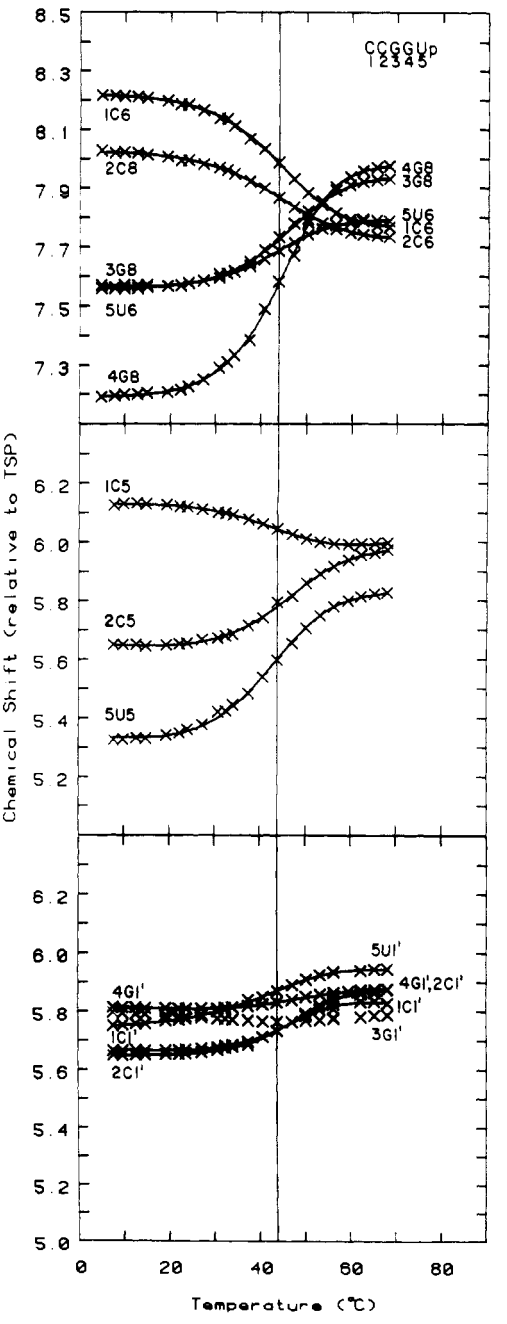

FIGURE 4: Chemical shift vs. temperature for nonexchangeable base and 1′ protons of 0.57 mM CCGGUp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Solid curves are fits of the data to eq 1. The vertical line is the average of the NMR T_m's.

spectively; $c_T = 2c_D + c_S$; K, ΔH, and ΔS are respectively the equilibrium constant and enthalpy and entropy changes for double-helix formation. From the fitted thermodynamic parameters, the melting temperature, T_m, for each proton can be calculated:

$$T_m = \frac{\Delta H}{\Delta S + 2.3R \log c_T} - 273.15 \qquad (4)$$

These T_m's are listed in Table III.

The temperatures used for the data points in Figures 1–5 were determined immediately before or after a spectrum, by measuring the chemical shift difference, $\Delta\delta$ (ppm), between the aliphatic and hydroxyl protons for neat methanol (below room temperature) or neat ethylene glycol (above room temperature). There is some disagreement on the appropriate calibration curve for this temperature measurement (Friebolin et al., 1979; Kaplan et al., 1975; Van Geet, 1968, 1970). We have used the calibration supplied by Bruker:

$$\text{methanol:} \quad T\ (°C) = -116.5\Delta\delta + 206.4 \qquad (5)$$

$$\text{ethylene glycol:} \quad T\ (°C) = -96.1\Delta\delta + 183.6 \qquad (6)$$

An alternative calibration for ethylene glycol has been determined by Friebolin et al. (1979) with the transition temperatures of seven liquid crystals:

$$\text{ethylene glycol:} \quad T\ (°C) = -102.3\Delta\delta + 193.9 \qquad (7)$$

For each oligomer, we have averaged the T_m's obtained with the Bruker calibration and then converted this temperature to the Friebolin scale. The differences range from 0.9 to 2.1 °C. Both average T_m's are listed in Table III.

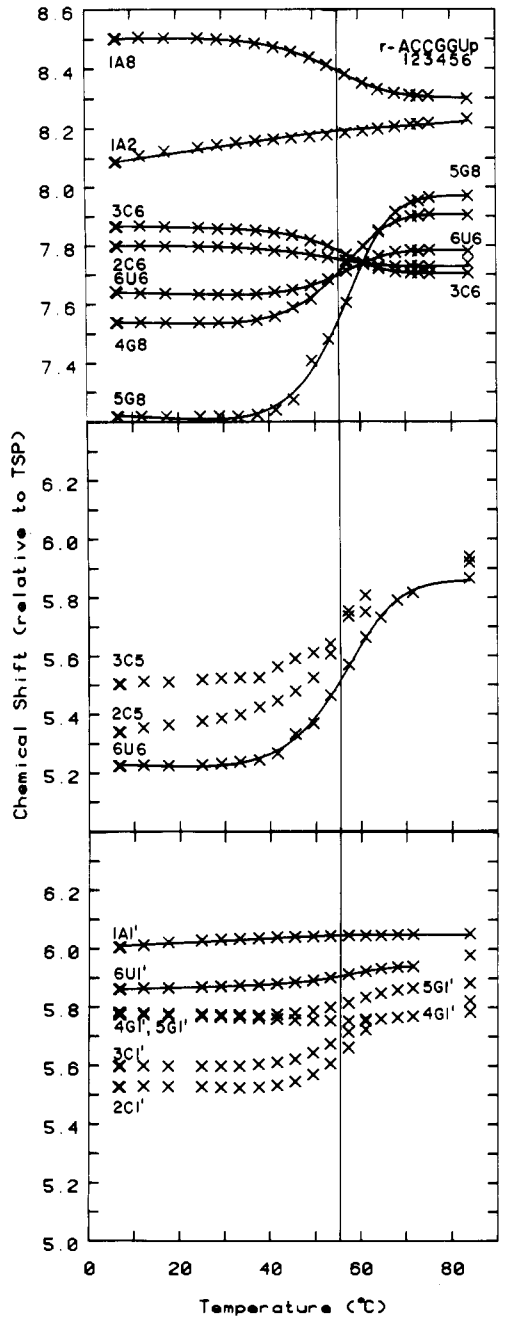

FIGURE 5: Chemical shifts vs. temperature for nonexchangeable base and 1′ protons of 0.41 mM ACCGGUp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Solid curves are fits of the data to eq 1. The vertical line is the average of the NMR T_m's.

The NMR melting temperatures can be compared with T_m's calculated for the NMR concentrations by using thermodynamic parameters [Table III of Petersheim & Turner (1983a)]. The T_m's predicted from the temperature-independent thermodynamics derived from optical melting studies in H_2O are listed in Table III. There is some question as to how much agreement is expected between optical and NMR T_m's. First, there is the ambiguity described above in measuring the temperature for NMR. Second, the T_m's are expected to be slightly different in H_2O and D_2O (Albergo et al., 1981). Third, Pardi et al. (1981) suggest NMR T_m's may appear higher than optical T_m's if the fast-exchange limit is not applicable at low temperature. This would cause deviation from simple population averaging of chemical shifts and result in a chemical shift closer to that of the double helix than

Table III: Melting Temperatures Derived from Oligomer ^{1}H NMR Melting Curves and Predicted from Optical Studies[a]

proton[c]	1C	2C	3G	4G
0.8 mM CCGGp T_m's (°C)[b]				
6/8	37.7	37.0	37.6	36.3
5	35.3	38.8		
1′	39.2	38.4	*d*	*d*
average NMR T_m = 37.5 ± 1.3 °C[b] (38.4)[e]				
predicted T_m = 34.6 ± 0.5 °C[f] (38.9 ± 1.3 °C)[g]				

proton[c]	1C	2C	3G	4G	5A
1.1 mM CCGGAp T_m's (°C)[b]					
6/8	52.8	52.8	52.4	51.8	50.9
5/2	49.3	53.3			52.5
1′	53.7	52.6	*d*	*d*	53.0
average NMR T_m = 52.3 ± 1.2 °C[b] (54.1)[e]					
predicted T_m = 55.3 ± 0.3 °C[f]					

proton[c]	1A	2C	3C	4G	5G
0.77 mM ACCGGp T_m's (°C)[b]					
8/6	(45.2)[h]	(46.6)[h]	46.6	45.7	45.0
2/5	(44.1)[h]	38.4	47.9		
1′	*d*	50.7	47.4	*d*	*d*
average NMR T_m = 45.8 ± 3.2 °C[b] (47.2)[e]					
predicted T_m = 47.9 ± 0.4 °C[f]					

proton[c]	1C	2C	3G	4G	5U
0.57 mM CCGGUp T_m's (°C)[b]					
6/8	43.3	43.1	45.0	44.0	42.4
5	43.5	44.5			41.2
1′	46.4	46.7	*d*	43.7	43.2
average NMR T_m = 43.9 ± 1.6 °C[b] (45.2)[e]					
predicted T_m = 46.5 ± 0.1 °C[f]					

proton[c]	1A	2C	3C	4G	5G	6U
0.41 mM ACCGGUp T_m's (°C)[b]						
8/6	53.7	53.8	56.4	56.4	56.4	55.8
2/5	*d*	*d*	*d*			55.8
1′	*d*	*d*	*d*	*d*	*d*	57.5
average NMR T_m = 55.8 ± 1.4 °C[b] (57.9)[e]						
predicted T_m = 58.2 ± 0.6 °C[f]						

[a] 1.0 NaCl, 10 mM cacodylate, 1.0 mM EDTA, pH 7 (prior to lyophilization), 99.8% D_2O. [b] Temperature determined from Bruker's calibration of ethylene glycol and methanol (eq 5 and 6). [c] Proton types are A8, A2, A1′, C6, C5, C1′, G8, G1′, U6, U5, and U1′. [d] T_m not obtained. [e] Temperature determined from the ethylene glycol calibration by Friebolin et al. (1979) (eq 7). [f] Predicted from temperature-independent thermodynamics in Table III of Petersheim & Turner (1983a). [g] Average of T_m's for CCGG in parentheses. [h] These resonances have small changes in chemical shift.

expected. Considering the various ambiguities, the NMR and optical T_m's are in quite good agreement.

Data concerning the $J_{1'2'}$ coupling constants are listed in Table IV. At low temperatures, the $J_{1'2'}$'s are less than 2.4 Hz, and most are too small to measure ($J_{1'2'} < 1$ Hz). At higher temperature, the 1′ resonances are split. For each oligomer, there is at least one 1′ resonance that splits at lower temperature than the others. The lowest temperatures at which these splittings were observed ($J_{1'2'} > 1$ Hz) are listed in Table IV. Except for ACCGGp, the 3′ ribose always splits first. For ACCGGp, the 5′C ribose splits first.

Figure 6 presents the temperature dependence of the line widths for the G8 and A8 protons in ACCGGUp. Similar plots are observed for the other oligomers and are available as supplementary material (see paragraph at end of paper regarding supplementary material). Errors are 5–10%. All the line widths decrease to less than 10 Hz at temperatures below the transition. For CCGG at 7 °C, the line widths of

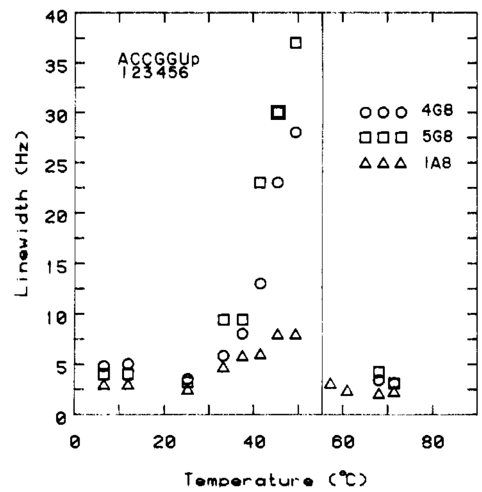

FIGURE 6: Line width vs. temperature for the 4G8 (○), 5G8 (□), and 1A8 (△) resonances of 0.41 mM ACCGGUp in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM cacodylate, pH 7, 99.8% D_2O. Line widths are full widths of half maximum height. Errors are 5–10%. The vertical line is the average NMR T_m. Spectra were obtained at 400 MHz.

the 3G8 and 4G8 protons are 10.4 and 12.0 Hz, respectively.

Discussion

The goals of this work are to provide details on the conformations and the single-strand to double-helix transitions of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. In particular, it is useful to know if the single-strand to double-helix transition is a two-state process and whether all these oligomers have similar conformations in the double-helical state. The results are important for interpreting thermodynamic measurements on these oligomers (Petersheim & Turner, 1983a).

One equilibrium that can cause deviations from two-state behavior is aggregation (Nelson et al., 1981). There are two general indications of aggregation in NMR experiments [e.g., Borer et al. (1975), Patel & Canuel (1979), and Freier et al. (1983)]: (1) broad and increasing line widths with decreasing temperature below the helix transition region and (2) inordinate sloping in low-temperature base lines. For example, with d-(GGAATTCC) at 5.3 mM, 0.1 M NaCl, 360 MHz, lowering the temperature from 15 to 5 °C, causes the line widths of the thymine 6 and thymine CH_3 5 resonances to broaden from 10 to 15 Hz and 6 to 10 Hz, respectively (Patel & Canuel, 1979). With $(dG\text{-}dC)_3$ at 400 MHz, the line widths of the GH8 resonances at 8 °C are 16 Hz under conditions producing aggregation [0.8 mM $(dG\text{-}dC)_3$, 1 M NaCl] but only 4 Hz in the absence of aggregation [1.4 mM $(dG\text{-}dC)_3$, 0.1–0.01 M Na^+, or 0.1 mM $(dG\text{-}dC)_3$, 1 M NaCl] (Freier et al., 1983). In those studies (Patel & Canuel, 1979; Freier et al., 1983), the chemical shifts of the terminal CH 5 and CH 6 protons are also strongly dependent on temperature, even below the single- to double-strand transition region. Typical slopes range from 0.006 to 0.023 ppm/°C. Similar effects are seen in other studies where aggregation is present (Borer et al., 1975; Arter et al., 1974).

The temperature dependence of the chemical shifts and line widths for the molecules in this study are presented in Figures 1–6. The slopes of the low-temperature chemical shifts for terminal CH 5 and CH 6 protons range from roughly 0 to 0.003 ppm/°C. For CCGGAp, ACCGGp, and ACCGGUp, the low-temperature line widths are all less than 7 Hz. For CCGGp and CCGGUp, the lowest temperature line widths are slightly less than 10 Hz, and they are decreasing with

Table IV: 1′–2′ Coupling Constant Data

ribose residue	CCGGp first doublet[a] ($J_{1'2'}$ in hertz)/ av NMR T_m	CCGGp high-temp $J_{1'2'}$ (Hz)/ high temp	CCGGAp first doublet[a] ($J_{1'2'}$ in hertz)/ av NMR T_m	CCGGAp high-temp $J_{1'2'}$ (Hz)/ high temp	ACCGGp first doublet[a] ($J_{1'2'}$ in hertz)/ av NMR T_m	ACCGGp high-temp $J_{1'2'}$ (Hz)/ high temp	CCGGUp first doublet[a] ($J_{1'2'}$ in hertz)/ av NMR T_m	CCGGUp high-temp $J_{1'2'}$ (Hz)/ high temp	ACCGGUp first doublet[a] ($J_{1'2'}$ in hertz)/ av NMR T_m	ACCGGUp high-temp $J_{1'2'}$ (Hz)/ high temp
5′A1′					>38	5			>45	5.4
C1′	>34	1.6	>32	3.9	17 (1.5)	4.9	>20	4.9	>45	
C1′	>34	4.0	>32	4.9	>38	5.4	>20	4.0	>45	
G1′	>34	6.0	>32	5.4	>38		>20	6.4	>45	
G1′	10.2 (2.0)	5.2	>32	4.9	35 (1.0)	4.9	>20	4.9	>45	
3′A1′/U1′			(8.5) (1.9)	4.9			(7.7) (2.4)	5.4	(6.6) (2.0)	6.8
temp (°C)	37.5	73	52.3	65	45.8	70	43.9	68	55.8	84

[a] Temperature (°C) from eq 6 at which $J_{1'2'} > 1$ Hz; if temperature is in parentheses, it was lowest temperature measured; other values in parentheses are $J_{1'2'}$ at the given temperature.

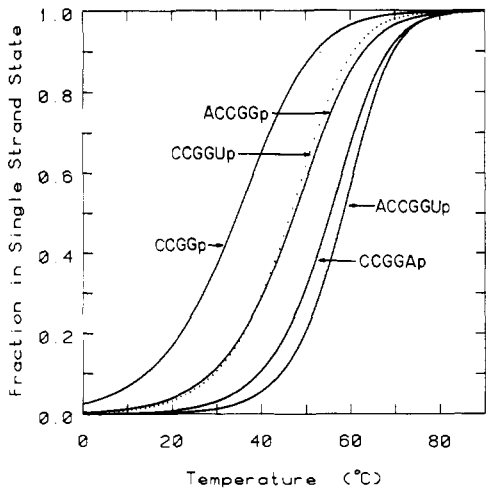

FIGURE 7: Degree of double-strand dissociation vs. temperature for 0.8 mM CCGGAp, 0.57 mM CCGGUp, 0.77 mM ACCGGp, 1.1 mM CCGGAp, and 0.41 mM ACCGGUp. Curves were calculated with temperature-independent thermodynamics [Table III of Petersheim & Turner (1983a)].

decreasing temperature. Thus there is no obvious evidence for aggregation.

The broadened line widths and temperature-dependent base lines that are observed probably are caused by processes other than aggregation. Two likely origins are the single- to double-strand transition and conformational flexibility in the helix. Figure 7 presents plots of the fraction of molecules single stranded vs. temperature for the oligomers. These curves were calculated for the NMR concentrations with the temperature-independent thermodynamics reported previously [Table III of Petersheim & Turner (1983a)]. CCGGp is still partially single stranded at 0 °C, which may account for some of the line broadening.

Another equilibrium that can cause deviations from two-state behavior is differential melting of terminal base pairs. Equivalence of T_m's for all base pairs in an oligomer has been considered evidence that differential melting is not important (Arter et al., 1974; Nielson et al., 1980; Pardi et al., 1981). Table III contains the T_m's measured in this study. The largest standard deviation of the T_m's for the protons in a given oligomer is the 3 °C observed with ACCGGp. However, the other deviations range from 1.2 to 1.6 °C. Moreover, there appear to be no consistent variations in the T_m's with the position of a proton in the helix. Similar results have been reported previously for CCGG (Arter et al., 1974). Evidently, differential melting is not important in these helices, and they dissociate cooperatively as a unit.

The conformations of the ribose residues in these oligomers can be deduced from the $J_{1'2'}$ coupling constants in Table IV. The fraction of the ribose population in the ^{3}E conformation for a given nucleotide can be estimated from (Altona & Sundaralingam, 1973; Evans & Sarma, 1974; Lee & Tinoco, 1980; Dhingra & Sarma, 1979; Lee et al., 1976)

$$\% \, ^3\text{E} \simeq 10(10 - J_{1'2'}) \quad (8)$$

Here $J_{1'2'}$ is in hertz. The only ribose 1′ resonances with $J_{1'2'} > 1$ Hz at the lowest temperatures measured are 5A1′ of CCGGAp, 5U1′ of CCGGUp, and 6U1′ of ACCGGUp. The splittings for these three resonances near 8 °C are 1.9, 2.4, and 2.0 Hz, respectively. Thus, about 80% of the population of each of these ribose groups is ^{3}E. All the other ribose residues appear to be 100% ^{3}E at low temperature. Since ^{3}E is the ribose conformation in A-form RNA, this is consistent with these oligomers having A-RNA structure (Arnott & Hukins, 1972; Borer et al., 1975; Cantor & Schimmel, 1980).

In principle, comparison of observed chemical shifts with those calculated for A RNA and other structures would give further insight into the conformations of these oligomers (Arter & Schmidt, 1976; Bubienko et al., 1981; Sarma et al., 1981). The chemical shifts in Figures 1–5 are particularly attractive for such comparisons since they are not perturbed by aggregation. We have made no attempt to calculate expected chemical shifts. However, qualitative inspection of the low-temperature data in Table II suggests the differences in chemical shifts for the CCGG cores of the various oligomers can be rationalized by additional shielding effects without invoking conformational changes. Thus at this qualitative level, there is no reason to suspect different conformations for the CCGG cores of these oligomers. Future advances in calculations of chemical shifts should make possible a more quantitative interpretation.

The predominance of NMR results are consistent with a two-state analysis in which the low-temperature state is similar to A-form RNA. However, some exceptions are detected. For example, the $J_{1'2'}$ coupling constant of at least one ribose 1′ resonance in each oligomer becomes greater than 1 Hz at lower temperatures than the others (see Table IV). This has also been observed for several double-helical oligomers by Alkema et al. (1981a,b). Apparently, specific ribose groups can exhibit temperature-dependent conformational heterogeneity while the oligomer is predominantly double helical. Except for ACCGGp, the 3′ ribose always splits at the lowest temperature. Thus, for double-stranded CCGGp, CCGGAp, CCGGUp, and ACCGGUp, the 3′ ribose has more conformational freedom than the other ribose residues. On the basis of NMR experiments on single-stranded dApdApdA, Olsthoorn et al. (1980) and Olsthoorn (1981) postulated greater conformational freedom for the 3′-terminal ribose in the single-strand helix state at low temperature. Recent X-ray structures of deoxyoligonucleotides provide evidence for anomalous behavior at the 3′ ribose of double helices (Dickerson et al., 1982). However, NMR results on single-stranded ribooligonucleotides suggest the 3′ ribose is more flexible for some sequences but not for others (Evans & Sarma, 1976; Cheng et al., 1980; Lee & Tinoco, 1980; Stone et al., 1981). In ACCGGp, the 5′C1′ resonance splits at the lowest temperature, and the 3′ ribose splits next. The 3′ ribose starts splitting 10 °C below the T_m, whereas in the other oligomers it splits more than 27 °C below the T_m. Thus addition of the 5′ A appears to increase the conformational freedom of the 5′ C ribose and decrease the freedom of the 3′ ribose.

The slopes of some of the chemical shift vs. temperature curves below the single- to double-strand transition region also suggest some conformational heterogeneity in the double helices (see Figures 1–5 and 7). Examples are the 1C5 of CCGGp, 1A2 and 2C5 of ACCGGUp, and 1A2, 1A1′, 2C5, 2C1′, and 3C5 of ACCGGp. The slopes of the lower base lines of these NMR melting curves seem unusually large compared with other resonances and with the degree of transition plotted in Figure 7. The comparison of ACCGGp with CCGGUp is particularly striking. These slopes may reflect temperature-dependent average conformations for these double strands. Once again, ACCGGp appears to have unusual conformational freedom.

Interestingly, the chemical shifts of the adenine and uracil resonances in CCGGAp and CCGGUp are not temperature dependent below the single- to double-strand transition region (see Figures 2 and 4). Moreover, the T_m's of these resonances

are close to the average T_m's for their respective oligomers. These results indicate the dangling adenine and uracil interact strongly with the CCGG helix and behave as part of the cooperative unit. The cooperativity observed for these dangling ends is much greater than that observed for single-strand stacking (Stone & Borer, 1981a,b; Stone et al., 1981; Lee & Tinoco, 1980; Pardi et al., 1981). In contrast, the adenine resonances of ACCGGp exhibit sloping low-temperature base lines and do not show cooperative melting behavior (see Figure 3). The results are surprising in that stacking of uracil is not considered to be particularly favorable (Warshaw & Tinoco, 1966; Ts'o et al., 1963; Ts'o, 1974; Gray et al., 1972), yet the 3′ U appears more stacked than the 5′ A. While surprising, the NMR results are consistent with the thermodynamic properties of these oligomers (Petersheim & Turner, 1983a). The thermodynamics indicate the increased double-helix stability due to a dangling end is associated with a more favorable enthalpy change for helix formation. Presumably, this reflects additional stacking interactions. The order of the oligomers with respect to the enthalpy contribution to helix stability is CCGGAp > CCGGUp > ACCGGp. Thus the thermodynamic data also indicate greater stacking for the 3′ U than the 5′ A.

These NMR studies have provided microscopic detail on the conformations and single- to double-strand transitions of CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. The results are qualitatively consistent with the oligomers having structures similar to A RNA at low temperature. The data may provide more rigorous structural determination when additional progress is made in calculation of chemical shifts (Sarma et al., 1981; Bubienko et al., 1981; Arter & Schmidt, 1976). The transition from the double-helix to single-strand state is essentially a two-state process. However, subtle local variations are observed that suggest conformational flexibility in some of the double helices. These are particularly evident with ACCGGp. Apparently, as suggested by Alkema et al. (1981a,b), the effect of adding a 5′ dangling nucleotide is considerably different from adding a 3′ dangling nucleotide. In general, the NMR results support the approaches used previously in deriving and interpreting the thermodynamics for these oligomers (Petersheim & Turner, 1983a).

Acknowledgments

We thank Dr. S. M. Freier for stimulating discussions, B. J. Burger for assistance with the analysis and figures, and Y. Kuroda for expert technical help with the NMR.

Supplementary Material Available

Four figures of NMR line widths vs. temperature (4 pages). Ordering information is given on any current masthead page.

Registry No. CCGGp, 83831-15-6; CCGGAp, 83831-16-7; ACCGGp, 83831-17-8; CCGGUp, 83831-18-9; ACCGGUp, 83831-19-0; CCGG, 55048-62-9.

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Nuclear Magnetic Resonance Study on the Exchange Behavior of the NH–N Protons of a Ribonucleic Acid Miniduplex†

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ABSTRACT: The exchange behavior of the guanine N(1) and uracil N(3) protons in the self-complementary hexanucleotide r(ApApGpCpUpU) has been studied at 5 °C in 80% H_2O/20% D_2O by proton NMR. Under these conditions, the hexanucleotide forms a stable miniduplex. The exchange rate of all Watson–Crick NH protons is unaffected by addition of trifluoroethylamine up to 0.07 M. On the other hand, addition of phosphate buffer, pH 6.9, enhances the exchange rate of the uracil N(3) protons of both terminal and internal A·U base pairs but does not influence the exchange rate of the guanine N(1) protons of the central G·C base pairs. Catalysis by increased phosphate concentrations results in an open-limited rate of the internal A·U base pairs with k_{ex} = 233 s^{-1}, equivalent to a lifetime of 4.3 ms. The proton exchange of the central G·C is regulated by the opening rate of the central core of the miniduplex. On the other hand, the sensitivity of the exchange rate of internal as well as of terminal A·U base pairs can be explained by their reduced lifetime due to end "fraying" and a subsequent catalysis of the exchange process from the opened state. These results suggest that it may be possible to probe labilized parts of RNAs such as tRNA by gradual addition of the exchange catalyst phosphate and to monitor their exchange rates by proton NMR.

The double-stranded, helical structure of DNA is known to exhibit thermally induced local fluctuations, the so-called "breathing" modes (Printz & von Hippel, 1968; McConnell & von Hippel, 1970a,b). Conformational fluctuations may be involved in biologically significant processes, including recognition of DNA base sequences by regulatory proteins in the course of DNA recombination, RNA transcription, and other processes of gene expression, as well as intercalation of drugs, mutagens, and carcinogens.

A number of techniques have been introduced to study the transient opening of double-helical DNA and RNA since the pioneering studies by Printz & von Hippel (1965) and Englander & Englander (1965). Among them, nuclear magnetic resonance (NMR) is a powerful method to obtain information not only on proton exchange rates but also simultaneously on the conformation and environment of the nucleic acids.

In a previous paper we presented a study of the proton exchange of uridine as a function of pH, temperature, and catalyst concentration (Fritzsche et al., 1981) completing the proton-exchange studies on monomeric units of nucleic acids done by McConnell and co-workers as well as other groups (McConnell & Seawell, 1972; McConnell et al., 1972; Cross, 1975; McConnell, 1978; Mandal et al., 1979). In this paper, we present ^1H NMR results on the proton-exchange behavior of a self-complementary hexaribonucleotide helix, r(ApApGpCpUpU)$_2$. The response of the NH–N resonances toward the addition of the potential catalysts phosphate and trifluoroethylamine (TFEA) demonstrates the existence of different mechanisms of the proton exchange for the three different base pairs of this RNA miniduplex. The results are discussed in terms of conformational fluctuations and end "fraying".

Materials and Methods

The hexanucleotide r(ApApGpCpUpU) was synthesized as described previously (Borer et al., 1975). The lyophilized sample was dissolved in phosphate buffer, pH 6.9. The final oligonucleotide concentration in 80% H_2O/20% D_2O was 1 mM (double strand). The starting concentration for the phosphate experiments was 10 mM phosphate. Additional phosphate buffer was added in lyophilized form. 2,2,2-Trifluoroethylamine hydrochloride (TFEA) was obtained from Aldrich Chemical Co. Small aliquots of a 0.16 M TFEA stock solution were added to the hexanucleotide solution containing 0.75 M NaCl, and the pH was adjusted to pH 7.3 after each addition.

The ^1H NMR spectra were recorded on two spectrometers, a Bruker WH-360 spectrometer located at the Mid-Atlantic NMR Facility Center, University of Pennsylvania, Philadelphia, PA, and a Bruker WM-500 spectrometer located at the Southern New England NMR Center, Yale University, New

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0006-2960/83/0422-0277$01.50/0