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Proton Nuclear Magnetic Resonance Studies of the Helix–Coil Transition of d-ApTpGpCpApT in D_2O Solution†

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ABSTRACT: The helix–coil transition of the self-complementary duplex d-ApTpGpCpApT between 5 and 70° has been monitored by following the nonexchangeable purine and pyrimidine resonances and the sugar H_1', resonances in D_2O, low ionic strength, pH 7. The resonances have been assigned on the basis of partial deuteration studies, from comparison of $T_{1/2}$ values and the temperature dependence of the chemical shift between 0 and 20°. The $T_{1/2}$ values increase in a sequential manner on proceeding from terminal to internal to central base pairs for the d-ApTpGpCpApT duplex. For a given base pair, the $T_{1/2}$ values are dependent on resonance position, and increase in the order pyrimidine 5 < sugar H_1' < purine 2. The differential melting curve of the central cytosine H_5 resonance yields a reaction enthalpy for the helix–coil transition of 34 kcal. The line width changes of the cytosine H_5 resonance at the $T_{1/2}$ of the helix–coil transition yields the kinetic parameters associated with the process in low ionic strength, D_2O, pH 7. The dissociation and formation rate constants are 0.65×10^4 sec^{-1} and 0.72×10^6 l. mol^{-1} sec^{-1}, respectively, at 41° and are consistent with the kinetic aspects of the helix–coil transition in related oligonucleotides determined from optical studies.

The helix–coil transition of the self-complementary duplex d-ApTpGpCpApT in H_2O solution has been investigated by monitoring the exchangeable Watson–Crick ring NH resonances by high-resolution proton nuclear magnetic resonance (NMR) spectroscopy. It was demonstrated that the hexanucleotide double helix opens in a sequential process from its ends (Patel, 1974a) (Figure 1A) and the thermodynamic parameters associated with the fraying of the individual base pairs were determined and interpreted (Patel and Hilbers, 1975). The studies were extended to describe the separation and characterization of the nucleation from the propagation reaction for helix formation and provide estimates of the kinetic parameters associated with the different steps (Hilbers and Patel, 1975). Further, a combination of proton and phosphorus NMR studies demonstrated that the phenoxazone ring of the peptide antibiotic actinomycin D intercalates between GC and CG central base pairs in double-helical d-ApTpGpCpApT in solution (Patel, 1974a,b). The above experiments were undertaken at low temperatures, conditions under which the double helix was predominantly intact, since at coil concentrations

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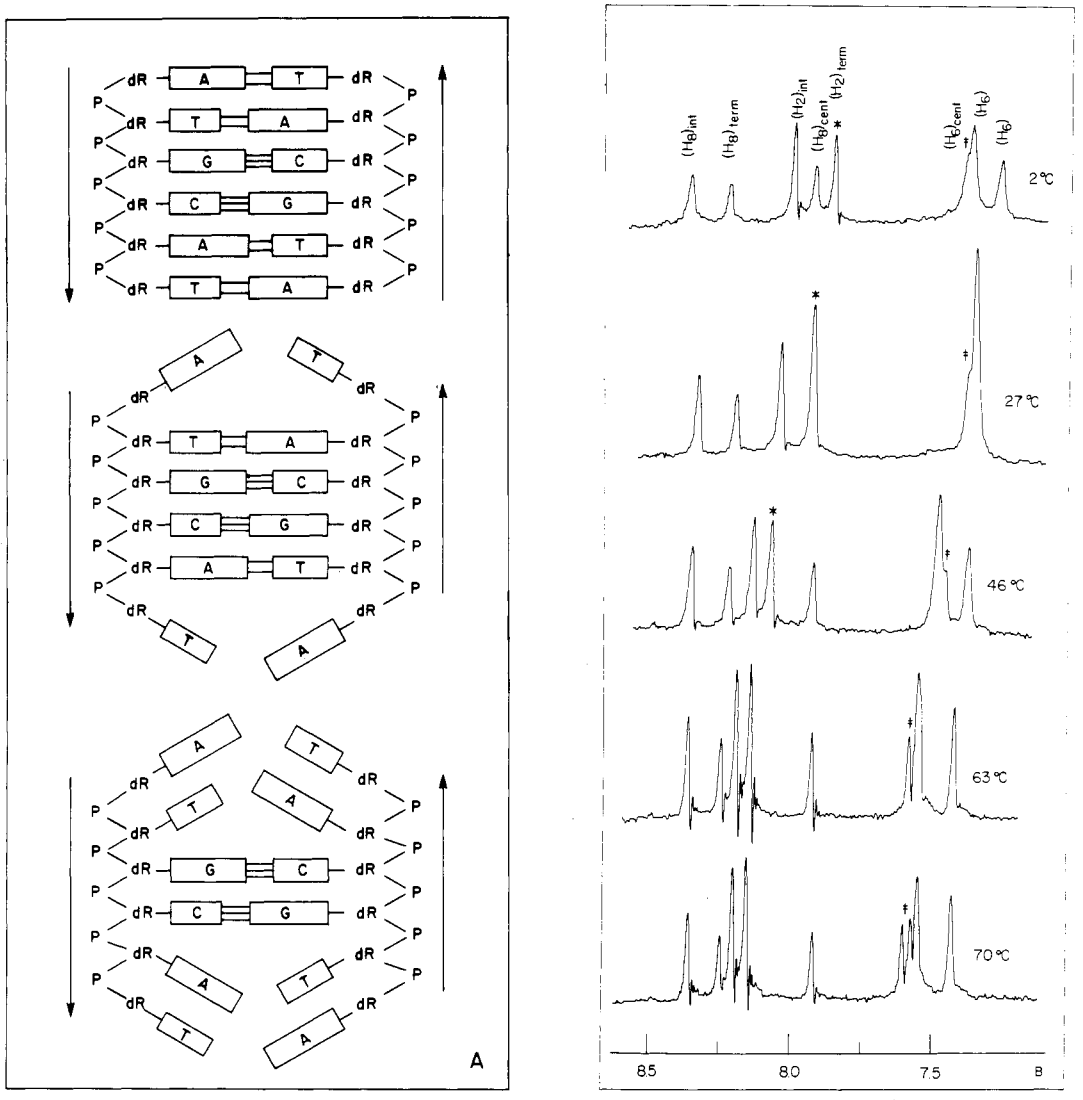


FIGURE 1: (A) Schematic illustrations of the hexanucleotide double helix with all base pairs intact, with terminal base pairs broken, and with terminal and internal base pairs broken. (B) The 270-MHz high-resolution proton NMR spectra (time average to improve signal to noise) of 9 m*M* d-ApTpGpCpApT in D_2O, low ionic strength, pH 7 between 7 and 8.5 ppm as a function of temperature. The purine H-8 protons were partially exchanged for deuterium by previous heat treatment. The terminal adenine H_2 resonance is designated by * and the central cytosine H_6 resonance is designated by ‡ so that they can be followed throughout the temperature range.

greater than 10% at the internal and central base pairs, the Watson–Crick ring NH exchangeable resonances broadened out and could no longer be followed.

An alternate proton NMR method monitors the nonexchangeable resonances of the purine and pyrimidine bases for oligonucleotides in D_2O solution over the entire helix–coil transition range (Cross and Crothers, 1971; Kreishman et al., 1974; Kan et al., 1974; Arter et al., 1974).

The thermodynamic and kinetic parameters from experiments on the nonexchangeable protons for d-ApTpGpCpApT in D_2O can be compared with related data obtained from the exchangeable ring NH Watson–Crick protons over a much narrower temperature range.

Experimental Section

The d-ApTpGpCpApT sample from Collaborative Research used in this study was repeatedly purified by passage through Sephadex G-25 and Bio-Gel P-2 columns. The samples are at low ionic strength since they contain no added salt. The NMR spectrum of the hexanucleotide contains no additional resonances from contaminant oligonucleotides and may be considered to be >95% homogeneous (Figure 1B). The samples were stable up to 70° since identical spectra (except for loss of intensity at the H_8 resonance due to exchange) were recorded prior and after heating to high temperature.

Spectra at 14 m*M* total strand concentration were recorded on a Varian HR-300 spectrometer interfaced with a Nicolet 1070 computer while spectra at 9 m*M* total strand concentration were recorded on a Bruker HX-270 spectrometer interfaced with a Nicolet-80 computer system. Sample temperatures are accurate to ±1° and are checked against methanol and ethylene glycol.

The spectra were calibrated relative to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)[1] as reference. We can rule out temperature-dependent interactions (Live and Chan, 1973) between standard DSS and the hexanucleotide at m*M* concentration, since the HOD peak (whose temperature dependence was previously known) was also used as a calibrant. Proton NMR studies of oligonucleotides in caco-

[1] Abbreviation used is: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

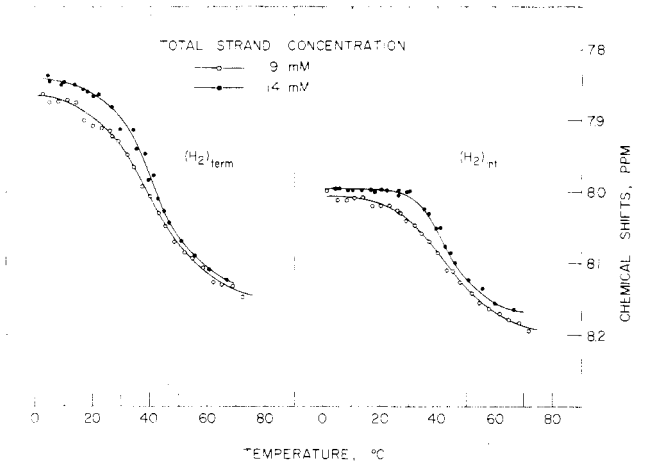


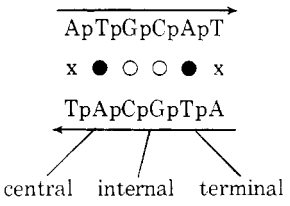
FIGURE 2: The temperature-dependent chemical shifts of the terminal and internal adenine H_2 resonances at 9 and 14 mM d-ApTpGpCpApT total strand concentrations. Solvent, D_2O, low ionic strength, pH 7.

dylate buffer containing DSS have indicated that the chemical shift difference between the methyl resonance of solvent cacodylate and standard DSS was independent of temperature in the presence of mM concentration of oligonucleotides (D. J. Patel, unpublished results).

Results

I. Temperature Dependence of Chemical Shifts. The 270-MHz high-resolution proton NMR spectra for 9 mM d-ApTpGpCpApT in D_2O solution, low ionic strength, and pH 7, as a function of temperature between 7 and 8.5 ppm, are presented in Figure 1B.

(A) Purine Protons. The d-ApTpGpCpApT sequence contains H_8 and H_2 protons from the terminal and internal adenines and an H_8 proton from the central guanine. Purine



H_8 resonances are known to exchange with D_2O at elevated temperatures (Schweizer et al., 1964). The 9 mM hexanucleotide sample was heated in D_2O prior to recording the spectra presented in Figure 1B. Three purine resonances between 7.7 and 8.5 ppm exhibit decreased area relative to the other two, so that the former are assigned to the H_8 protons and the latter to the H_2 protons.

Representative chemical shift–temperature dependence curves for the two adenine H_2 protons at 14 mM and 9 mM total strand concentration are presented in Figure 2. There are small chemical shift differences at low temperature as a function of concentration. The remainder of the manuscript will report on data accumulated at the lower (9 mM) concentration.

Earlier studies of the Watson–Crick ring N protons of the terminal and internal base pairs in d-ApTpGpCpApT demonstrated that the terminal base pair resonance exhibited a lower $T_{1/2}$ value and in addition exhibited a larger temperature-dependent chemical shift between 0 and 20° (Patel, 1974; Patel and Hilbers, 1975).

The H_2 resonance exhibiting a $T_{1/2}$ of 39.5° (14 mM) and 39° (9 mM) and a temperature-dependent chemical shift between 0 and 20° (Figure 2) is assigned to the adenine residue at the terminal base pair. The H_2 resonance exhibiting a $T_{1/2}$ of 42° (14 and 9 mM) and virtually temperature-independent chemical shifts between 0 and 20° (Figure 2) is assigned to the adenine residue at the internal base pair.

The two adenine H_2 resonances exhibit similar chemical shifts at 70°. The terminal adenine H_2 resonance shifts upfield by 0.275 ppm and the internal adenine H_2 resonance shifts upfield by 0.18 ppm on double helix formation at 5° (Figures 1B and 2).

The spectra in Figure 1 indicate that two purine resonances exhibit identical chemical shifts 7.9 ppm at ~30°. Since one of the resonances is a purine H_2 proton (designated by an asterisk) and the other resonance a purine H_8 proton with decreased area, it can be readily shown that a crossover of chemical shifts occurs as the temperature is varied through 27°. The chemical shift–temperature dependence curves for the five purine protons are plotted in Figure 3A.

The three H_8 resonances exhibit chemical shifts of 8.335, 8.225, and 7.91 ppm at 70° (Figure 3A). The guanine H_8 resonance can be readily identified (7.91 ppm) since the H_8 resonances of guanine occur at higher fields than those of adenine (see, Bovey, 1972). For the anti conformation about the glycosidic bond, the presence of a 5′-phosphate results in an ~0.1 ppm downfield shift in the purine resonance (Miller et al., 1971). On this basis, the H_8 resonance at 8.225 ppm is assigned to the terminal adenine (lacks a 5′-phosphate) and the 8.335-ppm resonance is assigned to the internal adenine (presence of a 5′-phosphate) in the spectrum at 70° (Figure 3A).

Both adenine H_8 resonances shift slightly upfield in the temperature range 70–30° and then slightly downfield in the temperature range 30–0°. The guanine H_8 resonance appears to be virtually temperature independent in the range 0–70° (Figure 3A).

(B) Pyrimidine Protons. The thymines at the terminal and internal base pairs each contribute a methyl resonance in the NMR spectrum between 1.3 and 1.8 ppm.

The methyl resonance exhibiting a $T_{1/2}$ of 31° and a large temperature-dependent chemical shift between 0 and 20° (Figure 3B) is assigned to the thymine residue at the terminal base pair. The methyl resonance exhibiting a $T_{1/2}$ of 36° and a smaller temperature-dependent chemical shift between 0 and 20° (Figure 3B) is assigned to the thymine residue at the internal base pair.

The two thymine methyl resonances are observed at 1.755 ± 0.01 ppm in the spectrum of d-ApTpGpCpApT at 70° (Figure 3B). On lowering the temperature to 5°, thereby causing double helix formation, the terminal thymine shifts upfield by 0.36 ppm while the internal thymine shifts upfield by 0.23 ppm.

The cytosine H_5 doublet occurs at 5.83 ppm at 70° and shifts upfield to 5.545 ppm on lowering the temperature to 34° (Figure 4A). Below 25°, it merges with the side bands of the HOD resonance and cannot be followed accurately. The cytosine H_5 resonance exhibits a $T_{1/2}$ of 41°.

The H_6 resonance from the two thymines and one cytosine in d-ApTpGpCpApT sequence are observed in the vicinity of 7.5 ppm. The cytosine H_6 is a doublet from coupling to the H_5 proton while the thymine H_6 resonances are singlets due to the presence of a CH_3 group at position 5.

The cytosine H_6 doublet is clearly visible in the 70° spectrum (denoted by ‡ in Figure 1B) and its temperature de-

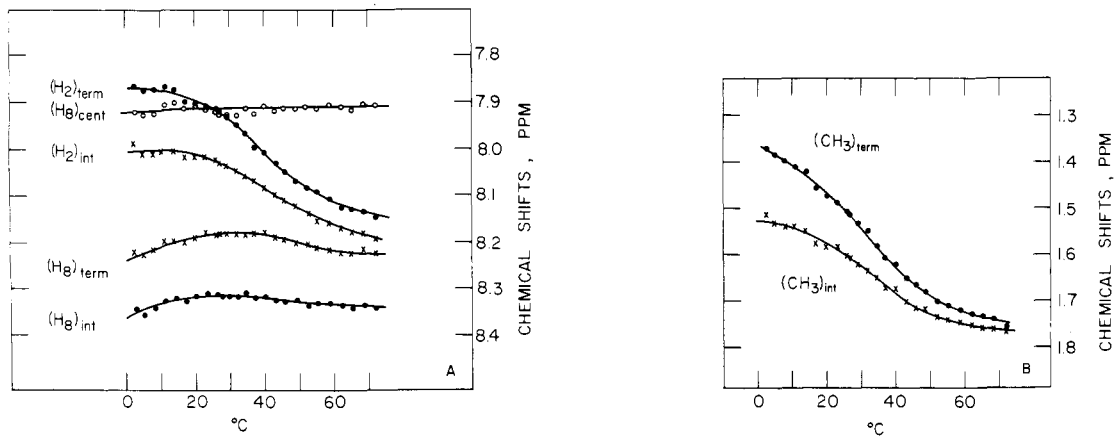


FIGURE 3: The temperature-dependent chemical shifts of (A) the purine H_2 and H_8 resonances and (B) the pyrimidine CH_3 resonances in 9 m*M* d-ApTpGpCpApT, D_2O, low ionic strength, and pH 7.

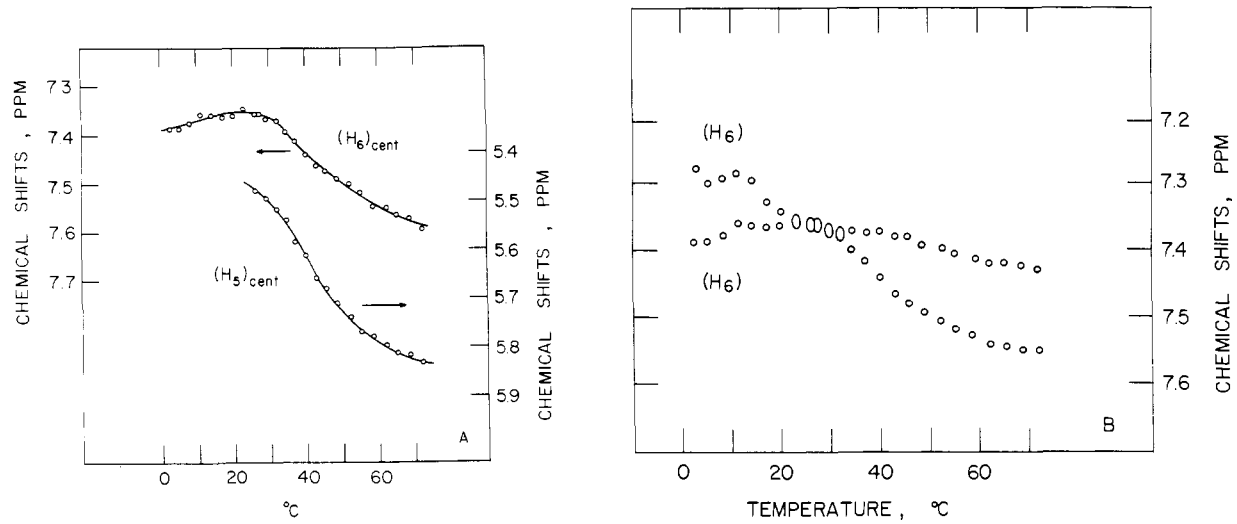


FIGURE 4: The temperature-dependent chemical shifts of (A) the central cytosine H_5 and H_6 resonances and (B) the thymine H_6 resonances in 9 m*M* d-ApTpGpCpApT, D_2O, low ionic strength, and pH 7.

pendence is plotted in Figure 4A. This resonance shifts upfield by 0.24 ppm in the temperature range 70–30° and then moves slightly downfield on lowering the temperature further to 0° (Figure 4A).

The unexpected nonequivalence of the thymine H_6 chemical shifts at high temperature and the ambiguity of tracing the titration curves at 27° where they merge (Figure 4B) do not permit differentiation and assignment of these resonances originating from the terminal and internal thymine residues.

(C) Sugar Protons. There are six sugar residues in the hexanucleotide sequence and the corresponding $H_{1'}$ triplets are observed in the spectra recorded as a function of temperature between 5.5 and 6.5 ppm (Figure 5A). All six triplets shift upfield as the temperature is lowered from 70 to 0°. The chemical shifts vs. temperature plots indicate merging of some of the triplets around 40° (Figure 5B), and the curves should be considered tentative at such temperatures until data at higher superconducting fields become available.

Resonances (3) and (6), which exhibit temperature-independent chemical shifts between 0 and 30° and $T_{1/2}$ values of 46 and 49°, respectively, are assigned to the sugar $H_{1'}$ resonances of the central base pairs. Resonances (4) and (5), which exhibit small chemical shift variations between 0 and 30° and $T_{1/2}$ values of 41 and 42°, respectively, are assigned to the sugar $H_{1'}$ resonances of the internal base pairs. Resonances (1) and (2) exhibit large chemical shift variations between 0 and 30° and $T_{1/2}$ values of 34.5 and 32.5°, respectively, and are assigned to sugar $H_{1'}$ resonances of the terminal base pairs. At this time we are unable to differentiate between the sugar $H_{1'}$ resonance associated with the purine base from that associated with the pyrimidine base at a given (terminal, internal, or central) base pair.

The $T_{1/2}$ values for protons at position 5 of the pyrimidine ring, position 2 of the purine ring, and 1′ of the sugar ring are summarized in Table I.

II. Thermodynamic Parameters. Gralla and Crothers (1973) have derived a simple expression relating the dissociation reaction enthalpy, ΔH, of the helix–coil transition to $T_{1/2}$, the temperature in K at the maximum of the differential melting curve, and $T_{3/4}$, the temperature above $T_{1/2}$ at which the magnitude of d(shift)/dT has fallen to half its maximum value:

$$\Delta H = \frac{4.37 \text{ cal}}{(1/T_{1/2}) - (1/T_{3/4})} \quad (1)$$

The differential chemical shift–temperature dependence curve of the central cytosine H_5 resonance which can be

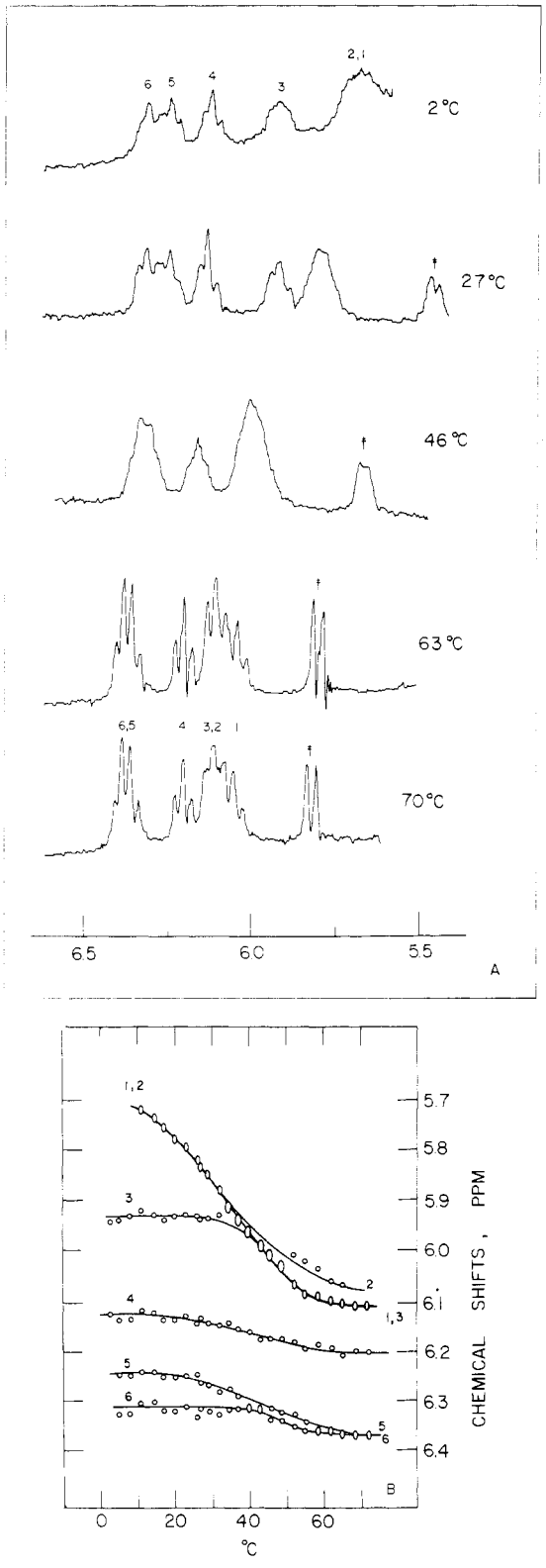


FIGURE 5: (A) The 270-MHz high-resolution proton NMR spectra (time averaged to improve signal to noise) of 9 m*M* d-ApTpGpCpApT in D_2O, low ionic strength, pH 7 between 5.5 and 6.5 ppm as a function of temperature. The ‡ designates the cytosine H_5 doublet. (B) The temperature-dependent chemical shifts of the six sugar $H_{1'}$ resonances designated 1 to 6 in 9 m*M* d-ApTpGpCpApT, D_2O, low ionic strength, and pH 7.

monitored over the temperature range 27–70° and can be unambiguously identified due to its doublet character (Figure 5A) was used in the calculation. This central base pair resonance monitors the transformation of the hexanucleotide double helix to strands. The helix–coil transition of d-ApTpGpCpApT is associated with a reaction enthalpy of 34 kcal/mol.

Table I: $T_{1/2}$ Values (°C) for the Terminal, Internal, and Central Protons at Positions 5, 2, and 1′ in 9 m*M* d-ApTpGpCpApT in D_2O, Low Tonic Strength, pH 7.

	Terminal	Internal	Central
Position 5	31[a]	36[a]	41[b]
Position 2	39	42	
Position $H_{1'}$	32.5	41	46
	34.5	42	49

[a] CH_3 group protons. [b] H_5 proton.

The equilibrium constant for the formation of a duplex from identical (self-complementary) strands is given by

$$K = \frac{f}{2(1-f)^2 C_T} \tag{2}$$

where f is the fraction of bases paired and C_T is the total oligonucleotide strand concentration. At $T_{1/2}$, $f = 1/2$, and the formation equilibrium constant simplifies to $1/C_T$. The equilibrium constants for the helix–coil transition of 9 m*M* d-ApTpGpCpApT solutions at $T_{1/2}$ (= 41°) are $K_{formation} = 110$ and $K_{dissociation} = 9 \times 10^{-3}$.

III. Kinetic Parameters. The central cytosine H_5 resonance in d-ApTpGpCpApT exhibits an 0.355-ppm chemical shift difference between 25 and 70°. This doublet broadens in the vicinity of $T_{1/2}$ for the helix–coil transition compared to its value at low and high temperatures.

Equation 3 describes the exchange of a proton between two sites for the condition of fast exchange with contributions from uncertainty broadening, where the helical state is designated site H and the coil state site C, with populations P_H and P_C, and lifetimes τ_H and τ_C.

$$\frac{1}{\pi T_2} = \frac{P_H}{\pi T_{2H}} + \frac{P_C}{\pi T_{2C}} + 4\pi P_H{}^2 P_C{}^2 (\nu_H - \nu_C)^2 (\tau_H + \tau_C) \tag{3}$$

The calculation was undertaken for the central cytosine H_5 resonance at the midpoint of the helix–coil transition, i.e., $P_H = P_C = 0.5$. The line width at 70° is 3.75 Hz and is assigned to $(\pi T_{2C})^{-1}$, the line width in the coil state. The line width at 2° is ~6.4 Hz and is assigned to $(\pi T_{2H})^{-1}$, the line width in the helix state.[2] The observed line width, $(\pi T_2)^{-1}$, at $T_{1/2}$ (= 41°), is ~7.5 Hz. The chemical shift difference, $(\nu_H - \nu_C)$, is assigned a value of ~100 Hz (0.36 ppm) for the central cytosine H_5 resonance.

Solving eq 3 with the above parameters yields $(\tau_H + \tau_C) = 3.08 \times 10^{-4}$ sec. Since $\tau_H = \tau_C$ when $P_H = P_C$, one obtains $\tau_H = \tau_C = 1.54 \times 10^{-4}$ sec. The rate constant for the unimolecular dissociation of the double helix to single strands is $k_{HC} = 1/\tau_H = 0.65 \times 10^4$ sec^{-1} at 41°.

In the previous section, the equilibrium constant for helix formation was determined to be 110 at $T_{1/2}$ (= 41°). The bimolecular rate constant for association of strands to form the double helix is given by $k_{CH} = K_{formation}\, k_{HC} = 0.72 \times 10^6$ l. mol^{-1} sec^{-1} at 41°.

[2] It is assumed that the $(\pi T_{2c})^{-1}$ value at 2° and $(\pi T_{2H})^{-1}$ value at 70° apply at the temperature of the rate measurement (41°).

Discussion

I. Fraying. The sequential increase in $T_{1/2}$ on proceeding from terminal to internal to central base pairs for d-ApTpGpCpApT in D_2O, low ionic strength, and pH 7 (Table I) may be compared with $T_{1/2}$ values of 34.5 and 44.5° elucidated for the terminal and internal base pairs from extrapolation of low temperature data for d-ApTpGpCpApT in H_2O, low ionic strength, and pH 7 (Patel and Hilbers, 1975).

II. Helix Dissociation. For both the terminal and internal AT base pairs, the thymine CH_3 resonance exhibits a $T_{1/2}$ value which is lower by 7 ± 1° than the value observed for the adenine H_2 resonance in the same base pair (Table I). This may reflect the greater tendency for the thymine base to unstack separately from the end of the helix on breakage of the Watson-Crick hydrogen bonds.

III. Reaction Enthalpy for Helix-Coil Transition. The differential melting curve for the H_5 resonance of the $(GC)_{central}$ base pairs yields a reaction enthalpy of 34 kcal for the helix-coil transition of d-ApTpGpCpApT, D_2O, low ionic strength, and pH 7. This value may be compared with a reaction enthalpy of 27.5 kcal for the helix-coil transition of related ribo-ApApGpCpUpU, in 0.05 *M* cacodylate, H_2O, and pH 7 (Pörschke et al., 1973). Borer et al. (1974) have reported reaction enthalpies of 37 and 34 kcal for the helix-coil transition of ribo-UpUpCpGpApA, and ribo-ApApCpGpUpU, respectively (see also Uhlenbeck et al., 1971).

IV. Rate Constants for Helix-Coil Transition. The linewidth data on the cytosine H_5 resonance of the $(GC)_{central}$ base pair have been utilized to determine the helix dissociation rate constant, k_{HC}, of 0.65×10^4 sec^{-1} (41°) for d-ApTpGpCpApT in D_2O, low ionic strength, and pH 7. This value may be compared with values of 12 (3.4°) and 4.5×10^2 sec^{-1} (23.3°) for ribo-ApApGpCpUpU in 0.05 *M* cacodylate, D_2O, and pH 7 by optical methods (Pörschke et al., 1973).

The bimolecular association rate constant for helix formation from separate d-ApTpGpCpApT strands, $k_{HC} = 0.72 \times 10^6$ l. mol^{-1} sec^{-1} (41°), may be compared with available literature data for oligoribonucleotides. For ribo-ApApGpCpUpU, recombination rate constants are 0.94×10^6 l. mol^{-1} sec^{-1} (7.6°) and 1.6×10^6 l. mol^{-1} sec^{-1} (23.3°) in 0.05 *M* sodium cacodylate (pH 7.0) (Pörschke et al., 1973).

The above parameters are consistent with the kinetic aspects of the helix-coil transition in nucleic acids (Applequist and Damle, 1965; Eigen and Pörschke, 1970; Craig et al., 1971; Crothers, 1969).

Acknowledgment

This research benefited from the expert technical assistance of Miss L. Canuel.

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