Nuclear Magnetic Resonance Study of Hydrogen-Bonded Ring Protons in Watson-Crick Base Pairs

(proton exchange/oligonucleotide melting)

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ABSTRACT We have studied the low-field proton magnetic resonance spectrum of the double-helical complex of d(A-A-C-A-A) with d(T-T-G-T-T). Well-resolved resonances for the thymidine N₃ and the guanosine N₁ protons are seen at 1°C, which broaden by 4°C and disappear above 9°C. The actual T_m of the complex was found to be 28°C by measurement of UV-absorbance change. There are several factors that could limit the observed exchange broadening of the resonance lines; the model that best fits the data is one in which the rate of double-helix dissociation limits the rate of exchange of the H-bonding protons with water.

In proteins (1) and nucleic acids (2, 3), the nuclear magnetic resonances (NMR) of certain hydrogen-bonded protons are well resolved at low fields. The proton NMR spectra of purified tRNA samples have shown particularly well-resolved resonances in the region between -11 and -15 ppm downfield from dimethylsilapentane sulfonate (DSS). There is considerable experimental support for assigning these resonances to the ring NH protons of the AU and GC base pairs of the cloverleaf model (2, 3). From the basic similarity of this spectral region in several purified tRNAs, as well as from their distinct differences, it has been clear that these resonances could be assigned to specific AU and GC base pairs. This has been done in recent studies by assuming an intrinsic position for the UN₃ proton in an AU pair and the GN₁ proton in a GC pair and by including the ring-current shifts from the nearest neighbors (4).

There are two points of critical importance in this analysis: the intrinsic position of the hydrogen-bonded protons must be known and the ring-current shifts must be calculated or derived from model compounds. One approach is to use tRNA and its fragments (5) as models, and derive a self-consistent set of parameters that allows the spectra to be predicted from the cloverleaf model. This analysis (4) relies on the calculations of Giessner-Prettre and Pullman (6) for the shape of the shielding field of the nucleotide bases, but includes an adjustable factor to scale its magnitude for a best fit.

In view of the potentially important applications of these measurements to nucleic acids of unknown secondary and tertiary structure, it seemed appropriate to determine the resonance position of these hydrogen-bonded protons in well-characterized model systems. Oligonucleotides can be synthesized with sufficiently simple sequences so that they form only

regular helices, thus avoiding unknown structural influences that may be present in tRNA. In this paper we show the spectrum we obtained for a double helix of two complementary pentanucleotides, and that, in fact, originally provided the information necessary to distinguish between AU and GC resonances in tRNA (3, 4).

A further point of general interest that has not been discussed is the temperature dependence of the NMR spectrum of the hydrogen-bonded protons. Observation of a high-resolution spectrum relies on slow exchange between these protons and those of the H₂O solvent. When the double helix is disrupted by heating, exchange is rapid and the resolved resonances are no longer seen. The problem to which we address ourselves here is how the onset of exchange relates to the physical disruption of the double helix. In other words, how is a melting curve detected by magnetic resonance related to a melting curve measured by absorbance change?

Earlier model systems for the intrinsic resonance position of the hydrogen-bonded protons were those studied by Katz and Penman (7) and by Shoup, Miles, and Becker (8), namely solutions of nucleosides in chloroform and (CH₃)₂SO. However, as pointed out by Katz and Penman, the AU pair does not hydrogen bond as strongly as the GC pair, and it was not possible to obtain the intrinsic position of the ring NH resonance by extrapolation to complete pairing. A more strongly bonded model system therefore seemed necessary.

The two pentanucleotides, d(A-A-C-A-A) and d(T-T-G-T-T), have been shown to form a complex in aqueous solution by high-resolution proton NMR measurements of the non-exchangeable protons at 100 MHz, and appeared to offer an excellent model system (9). The NMR spectra of the exchangeable proton resonance from -11 to -15 ppm of this pair of complementary pentanucleotides were measured at 220 MHz (Fig. 1). At 9°C a weak broad resonance is barely seen, while at 6°C it is clearly a single broad resonance with a full width at half height of about 500 Hz. As the temperature is lowered two peaks appear, which narrow further as the temperature is decreased until at 1°C the lower-field peak itself begins to split slightly. Note that at the intermediate temperature of 4°C both peaks are observed and each is broadened by about 40 Hz over the width at 1°C. Integration of the areas of the spectrum at 1°C shows that the lower-field peak is 4.1 ± 0.2 -times more intense than the upper-field peak. From these intensities it is very easy to see that the TN₃H resonance of the AT base pairs resonate at lower fields than the GN₁H proton. These results were considered in choosing the preliminary set of ring-current parameters (4), but some important interpre-

Abbreviations: NMR, nuclear magnetic resonance. DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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Fig. 1. NMR spectrum at 220 MHz of an equimolar mixture of d(A-A-C-A-A) and d(T-T-G-T-T) (sample preparation described in ref. 9). Phosphate buffer (about 0.1 M) (pH 7), about 0.5 M NaCl. The total concentration of each strand was about 0.04 M, estimated from the absorbance in a cell with a path length of 0.01 mm.

tive problems remain, such as the particular double-helix form of DNA present.

At the concentrations of oligomer and salt present in the NMR tube, the T_m measured by optical measurements is substantially higher than the temperature range over which the low-field resonances broaden and disappear. Fig. 2 shows a differential melting curve $[d(\text{absorbance})/dT]$ for the same sample whose NMR spectrum is recorded in Fig. 1. Measurements were made at 245 nm in a cell with a path length of 0.01 mm enclosed in a thermostatted housing. The thermodynamic parameters of the melting transition can be obtained from the shape of the differential melting curve. The maximum occurs at 30°C; at this point θ (the fraction of strands in double helix) is 0.414 (10). The heat of melting may be calculated from the equation (10)

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

where $T_{1/2}$ is 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 $d(\text{absorbance})/dT$ has fallen to half its maximum value. The heat obtained from Fig. 2 and Eq. 1 is 32 kcal/mol of double helix, in good agreement with the earlier estimate of 34 kcal/mol (9). The T_m in the present experiment (28°C, where $\theta = 0.5$) is higher than the previous value (9°C) (9) because the concentrations of oligomer and salt are higher than before.

Since the low-field proton resonances broaden and disappear (but do not shift) below the helix melting temperature, it is evident that the lifetime of the hydrogen-bonded protons is being detected under conditions where the helix is still thermodynamically stable. We assume that the line widths at 1°C have no contribution from the lifetime of the helix. At higher temperatures the exchange rate is given by $k_{\text{ex}} = \pi(\delta\nu - \delta\nu_0)$ sec⁻¹, where $\delta\nu$ is the observed width and $\delta\nu_0$ the width at 1°C, which for the single resonance at -12.4 ppm is 60 sec⁻¹. At 4°C both NMR peaks have a similar broadening of about 40 sec⁻¹ over the widths at 1°C, indicating that k_{ex} is about 125 sec⁻¹ for all five resonances. It is known from tritium-exchange experiments (11) that protons in double-helical DNA exchange with time constants several orders of magnitude slower than about 100 sec⁻¹ at 4°C. Hence exchange from the oligomer double helix must be occurring by transient opening of the helix in order to be so much faster than exchange from a longer DNA.

A simple general description of the exchange processes responsible for the line broadening is:

where the proton goes from the hydrogen bond in the helix (H) to either nonhydrogen-bonded (C) strand, from which state it may exchange rapidly with H₂O. For the GN₁H proton, the NMR line positions in the H and C states would be about -12.5 and -10.5 ppm, respectively. One explanation of the line broadening observed in the absence of a shift is that the lifetime of the proton in the helix is interrupted by being switched to the C state for a time that is long compared with the inverse of this NMR shift. Another is that the exchange C → H₂O takes place before it returns to H, in which case the lifetime in the helix is limited by chemical exchange of the proton with H₂O. As a subcase of this latter, H and C might be in fast exchange, weighed by the equilibrium strongly in favor of H, so that time-averaged shifts are too small to be observed, and the lifetime is interrupted by C → H₂O exchange. In this case there might well be differences between the exchange rates of the AU and GC protons. Further experiments could distinguish between these possible mechanisms.

Fig. 2. Differential melting curve of the sample whose NMR spectrum is shown in Fig. 1. Absorbance changes were measured over intervals of a few degrees at 245 nm (path length of cell was 0.01 mm) and divided by the temperature change to give $d(\text{absorbance})/dT$.

The net rate of the exchange with water, k_{ex} , can be limited either by the rate of helix dissociation, k_{-1} , or by the rate, $k_{\text{H}_2\text{O}}$. These two cases are differentiated by the relative magnitudes of $k_{\text{H}_2\text{O}}$ and $k_1 C_f$ (C_f is the concentration of each strand; note that helix formation is bimolecular). When $k_1 C_f \ll k_{\text{H}_2\text{O}}$, exchange occurs virtually every time the helix opens, and $k_{\text{ex}} = k_{-1}$. On the other hand, when $k_{-1} C_f \gg k_{\text{H}_2\text{O}}$, helix and coil interchange many times before exchange with water occurs, and $k_{\text{ex}} = k_{\text{H}_2\text{O}} \alpha$, where α is the fraction of strands in the coil form.

For several reasons it is likely that the rate of helix dissociation k_{-1} determines the rate of exchange in the particular system under study here. First, since the AU and GC resonances broaden together, it is plausible that the rate is limited by the same step, k_{-1} . It is, of course, possible that $k_{\text{H}_2\text{O}}$ is nearly the same for U and G, in which case $k_{\text{H}_2\text{O}}$ could still be rate-limiting.

More convincing is the argument based on the estimated magnitudes of the relevant rate constants. The lifetime of the double helix at various temperatures can be estimated from the T_m and the strand concentration. The stability of the particular pentanucleotide double helix under study is so low that relaxation measurements are impractical. However, a wide variety of oligomers (12, 13) show bimolecular association rate constants k_1 of about $10^6 \text{ M}^{-1} \text{ sec}^{-1}$, approximately independent of temperature. At T_m , the association equilibrium constant $K = 2/C_T$, where C_T is the total molar concentration of each strand. Since C_T is 0.04 M in the present sample, we calculate that at $T = T_m = 28^\circ\text{C}$, $k_{-1} \approx 2 \times 10^4 \text{ sec}^{-1}$, so that no high-resolution NMR peak could be observed.

To calculate the helix lifetime expected at low temperature, we use the estimate of zero activation energy for helix association and hence set the activation energy for k_{-1} equal to the heat of melting, or 32 kcal/mol. The Arrhenius expression then yields for k_{-1} at 4°C a value of 200 sec^{-1} , or a helix lifetime of 8 msec. Hence there is close agreement between the broadening of the resonances actually observed and that predicted from the kinetic properties of oligonucleotides, with the assumption that dissociation of the helix is responsible for the line broadening.

The observations reported here are typical of a helix-coil transition under the important limiting condition that the helix lifetime is much less than a few milliseconds at $T = T_m$. In this case the resonances of hydrogen-bonded protons should broaden and disappear at temperatures below $T = T_m$. Consider the diagram in Fig. 3, showing the helix-dissociation rate constant k_{-1} at temperatures below T_m ($k_{\text{H}_2\text{O}}$ is assumed not to be rate-limiting for exchange). When k_{-1} is smaller than the values in the shaded region (10^2 – 10^3 sec^{-1}), sharp resonances should be observed, which broaden and disappear as k_{-1} moves up through the shaded region. Comparison of lines *a*, *b*, and *c* shows the factors that determine how far below T_m the broadening should occur. For *a* and *b*, the helices have the same value of k_{-1} at T_m . However, *b* has a larger activation energy, implying that it contains more base pairs than *a*. In this case, sharp resonances should persist closer to $T = T_m$ for *b* than for *a*. For very high activation energies in *b*, the temperature at which the NMR lines broaden approaches T_m .

FIG. 3. Diagram to illustrate the factors controlling the broadening (lines *a*, *b* and *c*) or decrease in intensity (line *d*) of H-bonded proton resonances.

In *c* the helix dissociation rate is smaller at $T = T_m$, and broadening occurs closer to T_m than for *a*.

The other limiting possibility is that $k_{-1} < 10^2 \text{ sec}^{-1}$ at $T = T_m$, illustrated by line *d*. This result is found for some relaxations in tRNA (14). In this case the exchange between helix and coil is slow, and the resonance lines for the helix should remain sharp but diminish in intensity as the helix melts. The transition temperature observed by NMR and optical methods should agree in this limiting circumstance. For reactions similar to those of the present pentanucleotides, T_m is also a function of concentration so that in, principle, the three different cases (*a*, *c*, and *d*) could be obtained as a function of concentration. We expect that various examples of these cases will be observed in future studies of RNA melting by NMR methods.

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