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Kinetics for Exchange of Imino Protons in Deoxyribonucleic Acid, Ribonucleic Acid, and Hybrid Oligonucleotide Helices†

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ABSTRACT: The lifetimes for opening of individual base pairs in a DNA ($dCA_5G + dCT_5G$), an RNA ($rCA_5G + rCU_5G$), and a hybrid DNA–RNA ($rCA_5G + dCT_5G$) helix have been measured by proton nuclear magnetic resonance. The lifetimes were obtained by saturation recovery experiments performed on the hydrogen-bonding imino protons of the Watson–Crick base pairs. In these oligonucleotide helices the observed relaxation rates were dominated by exchange with water, with the magnetic spin–lattice relaxation time of the imino protons possibly being important only at the lowest temperatures in the DNA helix. It was shown that three interior base pairs in the DNA heptamer $dCA_5G + dCT_5G$ were in the open-limited region, which means that these imino protons exchange every time the base pair opens. The lifetimes of the terminal G·C base pairs in the DNA helix are much shorter than the interior A·T base pairs. The pH dependence of the terminal base pairs indicated that the ends of the helix open and close many times before exchange of the imino protons with water takes place. The temperature dependence of the lifetimes of the interior A·T imino protons in the DNA helix showed that these protons exchange only when the double helix has dissociated into single strands. Thus, these lifetimes measure the rate for dissociation of the double helix. The activation energy for this process was found to be 47 kcal/mol. Comparison of the lifetimes of the interior protons in the DNA, RNA, and hybrid helices showed that the rates of dissociation of the RNA and hybrid helices are very similar at 5 °C, whereas the rate for the DNA helix was approximately 1 order of magnitude smaller than that for the other two helices. The reasons for the differences in the kinetics of the three helices are discussed, as are the general dynamics of oligonucleotide helices in solution.

In double-helical nucleic acids the base-paired imino protons exchange slowly enough with water to be observed in nuclear magnetic resonance (NMR)[1] experiments. These protons were first studied in solution studies of tRNA and also in double-helical oligonucleotides [see Kearns (1977) and Robillard & Reid (1979) for reviews]. The chemical shift of an imino proton in a base pair depends upon its intrinsic shift and an interaction term that arises mainly from the ring currents of the neighboring base pairs in the sequence. As the interaction term is dependent upon conformation, most of the information obtained from the NMR of the imino protons of tRNAs and oligonucleotides has been used to study the conformation of these molecules in solution (Robillard & Reid, 1979; Kearns, 1977).

Crothers et al. (1974) used NMR to study the dynamics of *Escherichia coli* $tRNA^{fMet}$ by watching the broadening of the imino protons with temperature. Kinetic measurements obtained by this method are very limited in the range of lifetimes that can be observed. Johnston & Redfield (1977) applied saturation recovery experiments to the imino protons to determine the exchange rates with water of the individual imino protons in tRNA. Other workers used this technique to study the helix–coil dynamics of tRNA (Hurd & Reid, 1980). Early et al. (1981a,b) have recently used a long-pulse inversion recovery experiment to study the relaxation behavior of several DNA restriction fragments, and in a following paper we have used saturation recovery techniques to study the dynamics of several related DNA oligonucleotides in solution (A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results). Thus proton NMR is now beginning to show its usefulness in obtaining information on the dynamics, as well as the conformations, of nucleic acids in solution.

The kinetics of helix–coil transitions in oligonucleotides has mainly been studied by using relaxation techniques, such as temperature-jump experiments (Pörschke & Eigen, 1971;

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

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Ravetch et al., 1973). We decided to study the dynamics of oligonucleotides in solution by the use of NMR saturation recovery experiments on the hydrogen-bonding imino protons in double strands. The advantage of NMR over other relaxation techniques is its ability to resolve the kinetics of individual base pairs in a helix. The differences in the dynamics for many parts of the double helix can be monitored.

The exchange rates of individual imino protons with water in the following systems were studied: (1) a DNA helix, $dCA_5G + dCT_5G$, (2) an RNA helix, $rCA_5G + rCU_5G$, and (3) a DNA–RNA hybrid helix, $rCA_5G + dCT_5G$. We use the temperature dependence and pH dependence of these rates to obtain the dynamics of these helices in solution. The lifetimes for exchange for the imino protons on the interior of these helices were shown to reflect the lifetimes for dissociation of the double strands into single strands. Thus we are able to compare the dissociation rates of a DNA, an RNA, and a hybrid helix of the same sequence.

In a following paper we examine the exchange rates of individual imino protons in a dodecamer, dCGCGAATTCGCG, and two less stable analogues, one with a G·T base pair and one with an extra adenine base on one strand of the double helix. With the results presented in that work, as well as these studies on the DNA, RNA, and hybrid helices, we are able to obtain a detailed picture of the dynamics of base pair opening in double-helical oligonucleotides in solution.

Theory

We will outline the theory needed for understanding the NMR experiments described here; it follows closely the recent discussion of Johnston & Redfield (1981) on the theory of the exchange rates of imino protons measured by proton NMR.

For the saturation recovery experiments on the imino protons of nucleic acids the saturated peaks recover due to exchange with unperturbed water and by other relaxation processes such as the spin–lattice relaxation of the imino protons in the helix. The intensity of a peak varies as a function of time according to the equation

$$I(t) = I_0(1 - e^{-t/\tau_{obsd}}) \tag{1}$$

where I_0 is the equilibrium intensity and τ_{obsd} is the observed lifetime of the proton.

Proton exchange from hydrogen-bonded states in nucleic acids has been described by the scheme (Teitelbaum & Englander, 1975; Crothers et al., 1974; Kallenbach et al., 1976)

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_x[\text{cat}]} \text{solvent} \tag{2}$$

Here closed represents protons in a base-paired state, open represents a non-base-paired state, and k_x is the rate constant for the base-catalyzed exchange of the imino proton with solvent water. We will assume that there is no direct exchange of the imino proton with solvent water from the closed state.

The Bloch equations for exchange between three different environments have been described by Crothers et al. (1974), which were an extension of McConnell's two-state derivation (McConnell, 1958). The three-state scheme is simplified under certain conditions that have been described elsewhere (Hilbers, 1979; Crothers et al., 1974; Pardi, 1980). One important simplification under our conditions is that $k_x[\text{cat}] \gg 1/T_{1(\text{open})}$ where $T_{1(\text{open})}$ is the spin–lattice relaxation time of the imino proton in the open state. This means there is essentially no relaxation due to the T_1 of the open state. A second assumption, which will be discussed later, is that $k_x[\text{cat}] \gg k_{cl}$, which implies that every time a base pair goes from the closed state to the open state the imino proton exchanges with water. This has been referred to as the open-limited region (Hilbers, 1979; Kallenbach et al., 1976; Crothers et al., 1974). These two assumptions allow a two-state analysis of the system (Pardi, 1980); for the saturation recovery experiments this leads to

$$1/\tau_{obsd} = 1/\tau_{op} + 1/T_1 \tag{3}$$

where τ_{obsd} is the relaxation time in eq 1, τ_{op} is the lifetime for opening of the base pair ($1/k_{op}$), and T_1 is the spin–lattice relaxation time of the imino proton in the base-paired state.

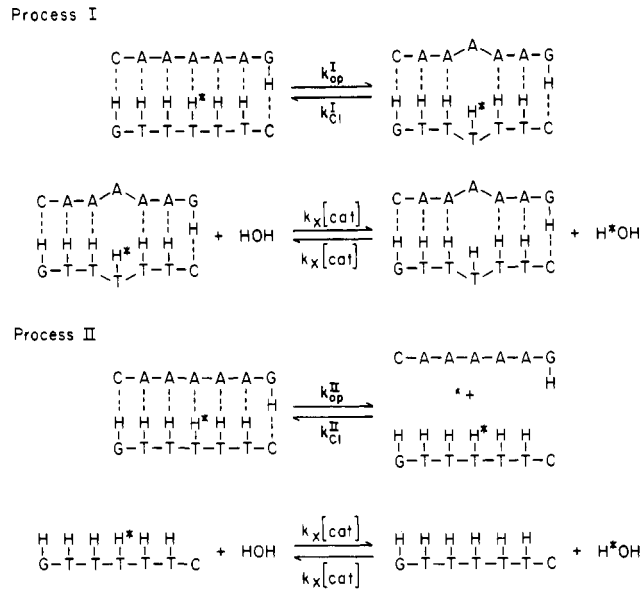

FIGURE 1: Schematic representation of exchange of imino protons with solvent water. Process I shows exchange by base-pair opening. Process II shows exchange by helix opening.

We will now describe the kinetics for the system when the exchange of the imino proton is in the open-limited region. For opening of one or more base pairs in a double strand, the open-limited region is where $k_x[\text{cat}] \gg k_{cl}^{I}$ (Crothers et al., 1974; Hilbers, 1979). Here k_{cl}^{I} is the rate constant for closing of an individual base pair in a double helix and is a unimolecular rate constant. The pathway for exchange of individual base pairs in a double helix is illustrated in Figure 1 and will be referred to as process I. Most of the studies to date on the rates of base-pair opening in double helices have dealt with helices where exchange of the imino protons probably went through this pathway (Englander et al., 1972; Kallenbach et al., 1976). However, in dealing with oligonucleotides it is possible that the open state, from which the exchange of the imino proton takes place, is the single strand. Process II in Figure 1 shows this pathway for proton exchange, where the double helix dissociates into single strands before exchange takes place. The kinetics of the double to single strand then enters into the exchange of the imino proton. If this is so, then the open-limited region occurs when $k_x[\text{cat}] \gg k_{cl}^{II}[\text{single strand}]$. Here k_{cl}^{II} is the association rate constant for the formation of a double strand from two single strands and is a bimolecular rate constant.

For either of the above cases it is possible to tell if exchange of the imino proton is open limited by changing the concentration of catalyst and measuring any change in the observed rate in the saturation recovery experiment. If the observed rate depends only on k_{op}, then it will not change with the concentration of catalyst. Proton exchange of the imino protons has been found to be open limited in tRNA studies (Hurd & Reid, 1980; Johnston & Redfield, 1981).

In process I, in addition to the open-limited region, it is possible for the kinetics to produce another limiting case called the preequilibrium region where $k_x[cat] \ll k_{cl}^I$. Crothers et al. (1974) and Hilbers (1979) have described the behavior of systems in this region and showed that $k_{obsd} = K_{open}k_x[cat]$ where k_{obsd} is the observed rate constant for exchange of the proton and K_{open} is the equilibrium constant between the open and closed states. This region has been described as fast exchange between the open state and closed state with exchange taking place by a "bleeding off" from the open state (Crothers et al., 1974). If the systems fall in this region, the equilibrium constants between the open state and closed state can be calculated from the observed rate of exchange, if estimates for k_x and the concentration of catalyst [cat] are known.

Kinetic Fraying vs. Equilibrium Melting. The end base pairs of oligonucleotides are known to have different properties from base pairs in the middle of a double helix (Patel & Hilbers, 1975). It has been observed that the imino protons of the terminal base pairs broaden at temperatures well below those of base pairs in the interior of a helix, and this effect has been used to help assign terminal base pairs in a helix. The broadening of a resonance accompanies rapid opening and closing of the base pair. We will use the term *kinetic fraying* to describe this behavior, in order to emphasize that the rapid opening and closing of the base pair is a kinetic phenomenon that depends upon the rates of conversion between the open and closed states. By contrast, the relative population of the two states is an equilibrium phenomenon. To describe the equilibrium behavior of a closed base-pair opening, we will use the term *equilibrium melting* of the base pair. This equilibrium melting depends only on K_{open} (which was defined above) and is equivalent to the term "melting" of a double strand used in describing the thermodynamics of nucleic acids (Bloomfield et al., 1974). The kinetics and equilibrium of base-pair opening are of course related by the equation $K_{open} = k_{op}/k_{cl}$. One can clearly see that there can be a large change in the kinetic fraying of the base pair, such as both the opening and closing rates increasing by 1 order of magnitude, with no change in the equilibrium melting of the base pair. We note that under most circumstances the proton NMR of the imino protons of nucleic acids monitors the kinetic behavior of the molecule.

Imino Protons Can Exchange by Several Different Processes. An important point to note is that even if exchange of the proton is in the open-limited region, this information is not enough to define the process from which exchange takes place. If there are several mechanisms for imino protons exchanging with H_2O, then the observed rate is the sum of the rates for the individual mechanisms.

We will assume that the exchange of an imino proton with H_2O requires the opening of the base pair as a primary step. This breaking of the Watson–Crick hydrogen bond may involve only a single base pair or may be a cooperative transition of part or all of the helix. By comparing the rates of exchange of individual base pairs in a helix, as well as the temperature dependence of these rates, it is possible to deduce the extent of cooperativity for exchange of the imino protons in a double helix. In the example discussed here we will assume that all protons are in the open-limited region. Let us consider two processes for exchange of the imino protons. The first involves exchange of individual base pairs, with each base pair being independent of the others, and will be referred to as base-pair opening, or process I. The second process is for exchange of the imino protons only from the single-stranded species with no exchange from the double strands, or partially formed double strands, and will be referred to as helix opening, or process II.

In process I the observed exchange rate will reflect the opening of individual base pairs and may be quite different for particular imino protons in a given sequence. Process II measures the rate of dissociation of the double strand into two single strands with the rates of all the imino protons being the same. In general the observed rate constant for exchange will be the sum of the rate constants for process I and process II. In the open-limited region the observed rate of exchange would be $k_{obsd} = k_{op}^I + k_{op}^{II}$. Using this fact and the Arrhenius equation, it is possible to derive the observed activation energy for exchange, which will be

$$\Delta E_{obsd} = \frac{1}{k_{op}^I + k_{op}^{II}}(k_{op}^I \Delta E_{op}^I + k_{op}^{II} \Delta E_{op}^{II})$$

where k_{op}^I and k_{op}^{II} are the rate constants and ΔE_{op}^I and ΔE_{op}^{II} are the activation energies for opening in processes I and II, respectively. This equation shows that the observed activation energy should be temperature dependent in the range where k_{op}^I and k_{op}^{II} are of the same order of magnitude. In the two processes described above the activation energy of the helix opening would be much larger than that for opening of an individual base pair, which means that as the temperature is increased, at some point, the helix-opening path will become more important. By studying the magnitude and temperature dependence of the lifetimes of the imino protons in oligonucleotides, it should be possible to detect whether exchange takes place by base-pair opening or helix opening. We note that many other processes such as cooperative opening of a particularly low-melting region of a double helix could also take place and the overall kinetics of exchange will involve all these paths.

Materials and Methods

The deoxyribooligonucleotides were synthesized by the diester method of Khorana (1968). The ribooligonucleotides were enzymatically prepared with polynucleotide phosphorylase (Martin et al., 1971). Separation and purification of the oligomers were done by RPC-5 column chromatography. Desalting of the samples was done in Bio-Gel P-2 columns. All samples were measured in 8.0 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.18 M NaCl, and 0.1 mM Na_2EDTA, pH 7.0, unless otherwise noted.

NMR spectra were taken on the HXS-360-MHz instrument at Stanford Magnetic Resonance Laboratory and the 270-MHz instrument at the Laboratory of Chemical Biodynamics. The temperature on both instruments was controlled to ±1 °C and was calibrated with methanol or by placing a thermocouple in an NMR tube while it was in the probe. Five-millimeter microtubes that hold 130–160 μL (Wilmad No. 508CP) were used in the experiments. Data were collected on a Nicolet 1180 computer with 8K data points and a sweep width of ±5000 Hz with the transmitter frequency set between 2300 and 2800 Hz downfield from water. The large water signal was attenuated by the Redfield 214 pulse (Redfield et al., 1975) and the use of either a low pass or tunable notch audio filter set on the water signal (Marshall et al., 1979), with the notch filter giving far superior results. All chemical shifts were referenced to the internal standard TSP.

Saturation of the imino protons was accomplished by a 100–200-ms pulse applied to the peak of interest, followed by a variable delay time before application of the Redfield probe pulse. Saturation of several peaks in the same region was accomplished by use of multiple decouplers or noise modulation

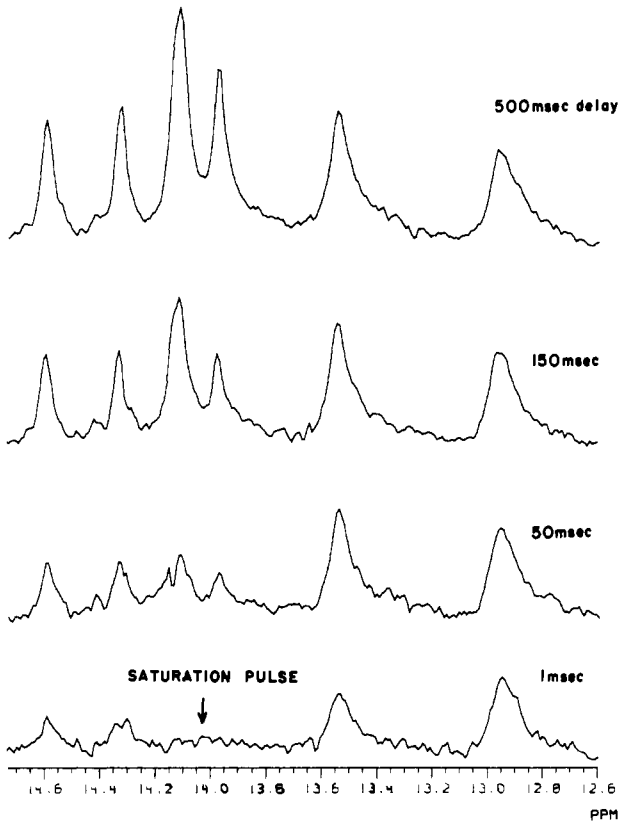

FIGURE 2: Spectra from saturation recovery measurement for DNA double helix ($dCA_5G + dCT_5G$) taken at 5 °C, pH 7. The arrow indicates the center of the saturation pulse. The saturation pulse was broadened by use of noise modulation (at 50 Hz) of the phase of the saturation pulse.

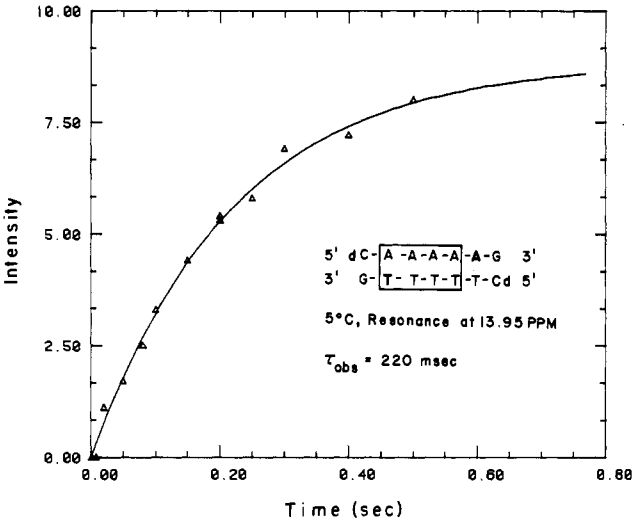

FIGURE 3: Saturation recovery experiment on an interior A·T resonance (base pair 2, 3, 4, or 5) at 13.95 ppm. The triangles are experimental data and the solid line is the best fit of a lifetime (τ_{obsd}) to the data with eq 1.

of the phase of the saturation pulse to broaden the width of the affected region.

Analysis of the lifetimes was done with a nonlinear least-squares fit to eq 1. Typically 10–15 different delay times were taken, with 500–800 scans for each point.

Results

The relaxation rates of the low-field Watson–Crick imino protons in the following double helices were studied: (I) $dCA_5G + dCT_5G$, (II) $rCA_5G + rCU_5G$, and (III) $rCA_5G + dCT_5G$ (which will be referred to as the DNA, RNA, and

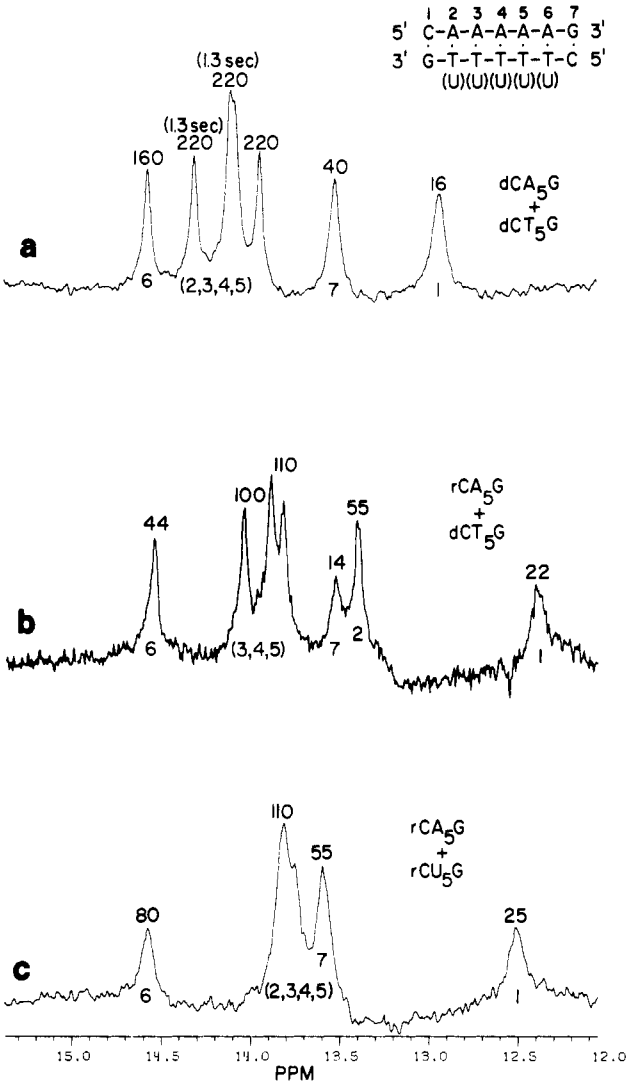

FIGURE 4: Observed lifetimes of imino protons at 5 °C, pH 7, for (a) DNA, (b) hybrid, and (c) RNA helices. For comparison two values are given in parentheses that were obtained by extrapolating to 5 °C from higher temperature data where the main relaxation mechanism is exchange by helix opening.

hybrid helices, respectively). The assignments of the imino protons as well as the temperature dependence of the chemical shifts have been previously reported (Pardi, 1980; Pardi et al., 1981). Saturation recovery experiments were used to measure exchange rates of the imino protons as well as the temperature dependence of these rates. An example of the data for $dCA_5G + dCT_5G$ is shown in Figure 2. An example of the fit of the results of a saturation recovery experiment to eq 1 is shown in Figure 3.

Rates of Exchange of Imino Protons in DNA Double Helix. Figure 4a shows the lifetimes of the imino protons of $dCA_5G + dCT_5G$ at 5 °C. Most of the numbers given were taken at 360 MHz, but others at 270 MHz showed no difference in the observed lifetimes. The peak at 14.1 ppm is due to two interior A·T imino protons and was analyzed as one peak that recovered with one lifetime; no biphasic behavior was observed in the fit of the lifetime of this peak. The terminal G·C base pairs (peaks 1 and 7 in Figure 4a) have much shorter lifetimes than the interior A·T resonances, and as the terminal imino protons are not in the open-limited region under our conditions, the observed lifetimes represent an upper limit on the lifetime for opening of the terminal base pairs. The observed lifetimes for the two terminal G·C base pairs differ by more than a

Table I: Lifetimes (Milliseconds) of Imino Protons of dCA_5G + dCT_5G in 28 mM Phosphate and 0.18 M NaCl, pH 7[a]

	C-A-A-A-A-A-G G-T-T-T-T-T-C 1-2-3-4-5-6-7						
base pair:	1	(2	3	4	5)	6	7
chemical shift:	12.93	13.9	14.1	14.1	14.3	14.6	13.6
temp (°C)							
5	16	220	220	220	220	160	40
10	8	180	150	150	210	120	19
13		100	90	90	130	60	
15	<5	80	70	70	80	45	12
17	*b*	36	46	46	58	20	*b*
20	*b*	15	15	15	15	⩽8	*b*

[a] The errors in these lifetimes are on the order of 15–25% with the larger errors observed for the shorter lifetimes (<25 ms). [b] The lifetime of this proton is too fast to measure at this temperature.

Table II: Comparison of Lifetimes of Imino Protons of dCA_5G + dCT_5G in 0.18 M NaCl and 28 mM Phosphate, pH 7.0, or 100 mM Phosphate, pH 8.0[a]

C-A-A-A-A-A-G
G-T-T-T-T-T-C
1-2-3-4-5-6-7

Terminal C·G Base Pairs at 5 °C		
position	1	7
chemical shift	12.9	13.5
lifetime (ms) at pH 7	16	40
lifetime (ms) at pH 8	<5	18

A·T Base Pairs at 15 °C					
position	(2	3	4	5)	6
chemical shift	13.9	14.1	14.1	14.3	14.6
lifetime (ms) at pH 7	80	70	70	80	45
lifetime (ms) at pH 8	70	50	50	75	30

[a] $[Na^+]$ = 0.22 M in the pH 7 buffer, and $[Na^+]$ = 0.3 M in the pH 8 buffer.

factor of 2 at 5 °C as seen in Figure 4a and Table I.

Temperature Dependence of Lifetimes of Imino Protons in dCA_5G + dCT_5G. The temperature dependence of the lifetimes of the imino protons in the dCA_5G + dCT_5G double helix is given from 5 to 20 °C in Table I. For the interior A·T protons the observed lifetimes increase from 20 to 10 °C where they level off. As will be discussed later, above 10 °C the lifetimes are measuring only the exchange of the imino proton, whereas below 10 °C the spin–lattice relaxation time (T_1) may also contribute to the observed lifetime. This behavior of the lifetimes of the imino protons has also been observed in longer, more stable molecules where at lower temperatures the T_1 of the imino protons in the helix dominates the measured rate and the exchange contribution is very small (Johnston & Redfield, 1978; Early et al., 1981a,b; A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results).

Lifetimes of Imino Protons in RNA and Hybrid Helices at 5 °C. Figure 4 shows the lifetimes of the imino protons in the three double helices at 5 °C. For the RNA and hybrid helices at this temperature the lifetimes are dominated by chemical exchange of the imino protons with H_2O, whereas the measured lifetimes for the DNA helix are at least partially due to the T_1 of the imino proton in the helix. The numbers in parentheses in Figure 4a represent the lifetimes for exchange of the imino protons extrapolated from the high-temperature data.

For the RNA double helix the resonance at 13.8 ppm contains the three interior A·U imino protons (no. 3–5) and was analyzed as one peak that fit well to a single exponential. The resonance at 13.6 ppm contains two imino protons, one from G·C no. 7 and one from A·U no. 2. The given lifetime is for the fit of this peak to a single exponential, because at the signal to noise available we were not able to fit this peak to a double exponential with any confidence. In the hybrid helix the two interior A·T resonances at 13.8 and 13.85 ppm recovered with the same lifetime and were analyzed as one peak.

Discussion

Are These Helices in the Open-Limited Region? An assumption that exchange of imino protons is open limited was made in the derivation used for the analysis of the saturation recovery experiments. The validity of that assumption is based on our experiments in which the concentration of the base was varied in the base-catalyzed exchange of the imino protons with H_2O and on other exchange studies of imino protons under similar conditions (Hilbers & Patel, 1975). As described earlier, the open-limited assumption requires that $1/\tau_x \gg 1/\tau_{cl}$ (see eq 2). Here $1/\tau_x = k_x[cat]$ where exchange is a base-catalyzed reaction, with the catalyst in our buffer being HPO_4^{2-} and OH^-. If changing the concentration of phosphate buffer or hydroxyl ions does not affect the observed rate, then the opening of the base pair is the rate-limiting step. Table II shows the lifetimes for the imino protons in the DNA helix at 28 mM phosphate, pH 7, and 100 mM phosphate, pH 8, at 5 and 15 °C. One sees that there is a large effect on the lifetimes of the terminal G·C imino protons when the concentration of base is increased, but there are much smaller changes in the lifetimes of the A·T base pairs with a change in the base concentration. The two A·T resonances at 13.95 and 14.3 ppm in the DNA helix show no measurable change in their lifetimes by increasing the concentration of base (see Table II). The lifetime of the penultimate A·T base pair no. 6 at 14.6 ppm changes from 45 to 30 ms in going from the buffer at pH 7 to that at pH 8 at 15 °C. This indicates that this base pair is not in the open-limited region at this temperature. We were not able to assign the other penultimate A·T imino proton in this helix to a specific resonance in the spectrum (Pardi, 1980), so one of the other four A·T peaks represents this proton. The peak at 14.1 contains two A·T protons, and the lifetimes of this peak change from 70 to 50 ms in going from the buffer at pH 7 to that at pH 8. It is possible that the two peaks at 14.1 ppm recover with slightly different lifetimes, but at the signal to noise of these data we observed no measurable biphasic behavior. The penultimate A·T base pair no. 6 is not in the open-limited region, and it is possible that the other penultimate base pair has a similar behavior. Thus the lowering of the lifetime of the two resonances at 14.1 ppm upon changing the concentration of base may be due to one of these peaks not being in the open-limited region, with this resonance tentatively assigned to the penultimate A·T base pair no. 2.

The terminal G·C base pairs are not in the open-limited region; thus their observed lifetimes represent only an upper limit to opening, and these base pairs may open and close many times (kinetically fray) before their imino protons exchange with water.

Activation Energies for Opening of Imino Protons in DNA Helix. Figure 5 shows an Arrhenius plot of the interior A·T proton at 14.3 ppm of dCA_5G + dCT_5G. The high-temperature behavior of the lifetime reflects changes in the rate of exchange of the imino proton with temperature, as will be discussed in a later section. A straight line can be drawn through the points from 13 to 20 °C, indicative of a constant

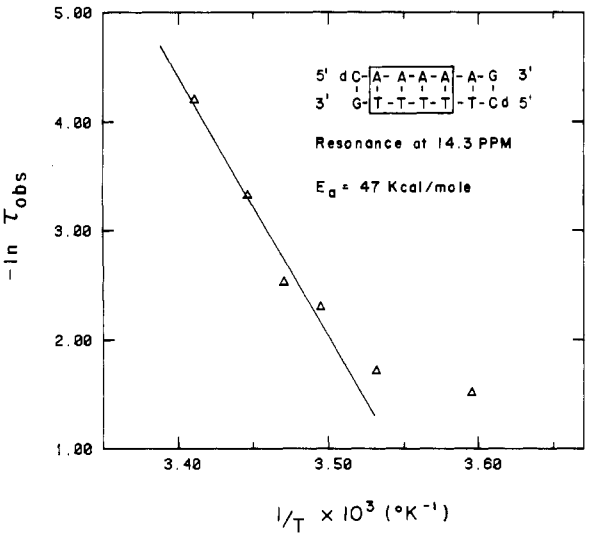

FIGURE 5: Arrhenius plot of an interior A·T proton (base pair 2, 3, 4, or 5) at 14.3 ppm of DNA helix. τ_{obsd} is the observed lifetime as defined in the text. Extrapolation of this line to 5 °C will give the lifetimes in parentheses in Figure 4.

activation energy. The activation energy for this process is 47 kcal/mol. The same activation energy is obtained for all three interior A·T imino protons, within the experimental error of ±15%.

The fact that two or more adjacent protons have the same activation energies for exchange could be due to several possibilities: (1) The protons exchange together, and the measured activation energy is for a cooperative process. (2) They exchange independently but have the same kinetics for opening of a single base pair. (3) They exchange independently and have different rates for a given temperature but the same activation energy for exchange. In the DNA helix the interior A·T base pairs have the same rates for exchange and the same activation energy, which puts this helix in case 1 or 2.

The activation energy of an individual A·T base pair opening has been measured in a twelve base pair restriction fragment (Early et al., 1981a,b) and the double helix dCGCGAATTCGCG (A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results) to be 14–16 kcal/mol. The 47 kcal/mol activation energy for the DNA helix measured here indicates a higher energy process than opening of an individual base pair. This makes the cooperative process the most likely explanation for exchange of the interior A·T imino protons in the DNA helix. In this case the cooperative process is the helix–coil transition. Further evidence to support this conclusion comes from temperature-jump measurements of the kinetics of the helix–coil transition in the dCA_5G + dCT_5G double helix. The lifetime for dissociation of the helix at 1 M NaCl (instead of the 0.18 M NaCl used in this work) has been measured by temperature-jump kinetics to be 167 ms at 10 °C with an activation energy of 43 kcal/mol (Nelson & Tinoco, 1982). These results are in good agreement with those found in this study and support the notion that the lifetimes of the interior A·T protons represent the lifetimes for dissociation of the double strand to single strands as outlined in process II, the helix-opening mechanism.

Contribution of Chemical Exchange vs. Spin–Lattice Relaxation to Lifetimes of Imino Protons. The relaxation rates of the imino protons have been studied in the tRNA (Johnston & Redfield, 1977, 1978, 1981) and also in several DNA oligonucleotides (Early et al., 1981a,b; A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results). There may be many processes involved in the relaxation of these protons. For example, spin–lattice relaxation can contribute to the rate as well as chemical exchange. Saturation recovery or inversion recovery experiments cannot distinguish between these relaxation mechanisms. Johnston & Redfield (1977) were the first to study the relaxation of the imino protons in tRNA and saw two types of behavior; at higher temperature the relaxation seemed to be exchange dominated, whereas spin–lattice relaxation was the important mechanism at lower temperatures.

For the interior A·T imino protons of the DNA helix, the four high-temperature points are in the region where exchange of the imino protons by helix opening is the main relaxation mechanism, but by 5 °C some other mechanism with a different activation energy has become important (see Figure 5). Spin–lattice relaxation and process I, base-pair opening, are the most likely relaxation processes at this temperature. Extrapolation of the high-temperature behavior gives the lifetime for dissociation of the double helix at 5 °C to be 1.3 ± 0.3 s. The contribution due to T_1 can then be calculated from the observed lifetime of 220 ms and would be 260 ms, on the assumption that process I is not important. This number represents a lower limit on the T_1 of the helix at this temperature since process I could be contributing to the exchange behavior at this temperature and thereby causing the lifetime for exchange to be lower than the 1.3 s calculated above. Modeling the behavior of the system, ignoring the spin–lattice relaxation time, indicates that base-pair opening (process I) would be the dominant exchange process in the DNA helix below 1 °C, on the assumption that the rates of opening of an individual A·T base pair in the interior of this helix are the same as those observed in longer deoxyoligonucleotide helices (A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results). So the observed lifetime of 220 ms at 5 °C for this helix may be partially due to process I as well as the contribution from the T_1 of the helix, but above 10 °C it is clear that the observed lifetime monitors only the dissociation lifetime of the DNA helix.

Comparison of Lifetimes in the Three Helices. The lifetimes of the interior A·U or A·T imino protons in the RNA and hybrid helices are much shorter than the lifetimes observed for the DNA helix at the same temperature. The equilibrium dissociation constants for the RNA and hybrid helices have been shown to be less than that found in the DNA helix (Martin & Tinoco, 1980), so it is likely that the lifetimes for dissociation of the two less stable helices are shorter than that of the DNA helix. Since the observed lifetime of the DNA helix was shown to measure the helix–coil transition (process II), we expect that this path would be even more important for the less stable RNA and hybrid helices. Therefore the lifetimes for exchange of the interior imino protons reflect the kinetics of the double to single strand transition in all three helices and can be used to compare the rates of dissociation of DNA, RNA, and hybrid helices of the same sequence.

For the RNA helix we have only a limited amount of data at temperatures other than 5 °C. The lifetimes of the interior A·U protons (peak at 13.8 ppm in Figure 4) were found to be 65 ms at 10 °C. The helix dissociation lifetime with temperature-jump measurements was found to be 45 ms at 10 °C (Nelson & Tinoco, 1982). This short lifetime for helix dissociation shows that the NMR lifetime is measuring the exchange lifetime and that T_1 is not important at this temperature. We expect that at 5 °C the lifetimes of the interior imino will still be dominated by exchange with the contribution from T_1 probably small. Since the RNA and hybrid helices have similar behavior at 5 °C (see Figure 4), we assume that

the lifetimes of the interior imino protons in both helices are mainly due to exchange and thus measure the dissociation lifetimes of these helices.

The lifetimes of the interior A·U or A·T imino protons (no. 3–5) in the RNA and hybrid helices are very close at 5 °C, indicating that the rates of dissociation of these helices are similar. The lifetimes for dissociation of the DNA helix were extrapolated from the high-temperature data to be 1.3 s at 5 °C. This means that the rate of dissociation of the DNA helix is 1 order of magnitude less than that in the RNA or hybrid helix of the same sequence. The reason for this large difference is not known, but the dissociation constant for the DNA helix $dA_8 + dT_8$ has been found to be over a factor of 2 smaller than that for the RNA helix $rA_8 + rU_8$ (Drobnies, 1979). In a comparison with other DNA and RNA oligonucleotide helices, the dissociation rates were sometimes larger for the DNA helix (Drobnies, 1979); further studies on the kinetics of RNA and DNA helices may help explain the sequence dependence of their rates of dissociation.

The pH dependence of the observed lifetimes of the terminal base pairs in the DNA helix (Table II) shows that these base pairs are not in the open-limited region. The lifetimes of the terminal base pairs on the RNA and hybrid helices are similar to those in the DNA helix (see Figure 4), and therefore they are most likely not in the open-limited region. This means that these base pairs open and close many times before their imino protons exchange with water. In the limit of $k_{cl} \gg k_x[\text{cat}]$, as discussed under Theory, the observed overall rate for exchange of the imino protons would be $k_{obsd} = K_{open}k_x[\text{cat}]$ (Hilbers, 1979). The rate constant for catalysis by HPO_4^{2-} has been measured by Hilbers & Patel (1975) in the oligonucleotide dATGCAT to be approximately 5×10^4 M^{-1} s^{-1} at 5 °C, pH 7, so for the same conditions in our buffer, $k_x[\text{cat}] = 5 \times 10^2$ s^{-1}. From the observed lifetimes of 16 and 40 ms for the G·C no. 1 and no. 7 imino protons, respectively, in the DNA helix at pH 7 (see Figure 4a), K_{open} for these base pairs is calculated to be on the order of 10^{-1}–10^{-2}, if we assume the preequilibrium condition. These base pairs are therefore mainly in the closed state with little equilibrium melting at this temperature. This is consistent with melting curves of chemical shifts vs. temperature found for the terminal base pairs of these molecules (Pardi et al., 1981).

Summary and Conclusions

The terminal base pairs in oligonucleotide double helices were found to have very different dynamics from base pairs in the interior of a helix. The terminal base pairs undergo kinetic fraying, which means there is very rapid opening and closing of the base pair. The rates of this kinetic fraying are dependent upon sequence as well as temperature.

The exchange rates of the imino protons of the interior base pairs were measured in the double helices $dCA_5G + dCT_5G$, $rCA_5G + rCU_5G$, and $rCA_5G + dCT_5G$ and were found to be in the open-limited region. This means that every time the base pairs opens, the imino proton exchanges with water, and so the exchange rates measure opening of these base pairs. Opening of these base pairs was found to take place only when the double strands dissociated into single strands. The dissociation rate constants of the RNA ($rCA_5G + rCU_5G$) and hybrid ($rCA_5G + dCT_5G$) helices were very similar at 5 °C and much larger than the rate constant for the more stable DNA helix.

By measuring the temperature dependence of the opening rates of the interior base pairs, we obtained the activation energy of 47 kcal/mol for helix opening in the DNA double strand. This energy can be very useful in defining the mechanism by which exchange of an imino proton takes place. The activation energy for individual base pair opening has been measured by NMR on DNA restriction fragments by Early et al. (1981a,b,) and on a DNA dodecamer by A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr. (unpublished results) to be 14–16 kcal/mol. The value of 47 kcal/mol measured in the $dCA_5G + dCT_5G$ helix thus corresponds to a much higher energy process than individual base-pair opening and was shown to represent helix opening.

Hurd & Reid (1980) have measured the opening rates of base pairs in the amino acid acceptor stem of partially unfolded *E. coli* $tRNA^{Phe}$. They were limited in their studies of the temperature dependence of these rates because the spin–lattice relaxation rates of the imino protons also contributed to the observed rates at lower temperatures. Thus they had only limited data with which to obtain activation energies of base-pair opening in the amino acid acceptor stem. This stem consists of five G·C pairs and one A·U base pair. They found an approximate value of 49 kcal/mol for one of the interior G·C base pairs in the stem and a qualitative trend to higher activation energies toward the interior of the helix. The approximate value of 49 kcal/mol can then be interpreted as measuring a cooperative opening of all or part of this helical stem. If this activation energy were found to be constant over a reasonable temperature range, then it would most likely arise from a single process, and the magnitude of this energy would help define that process.

Johnston & Redfield (1981) have also recently made an extensive study of the unfolding of several tRNAs by observation of the exchange rates of the imino protons. In yeast $tRNA^{Phe}$ in zero Mg^{2+} they were able to measure the exchange rates of several imino protons that had large activation energies. These activation energies were qualitatively interpreted as monitoring tertiary structural melting. The activation energies of other imino protons were used to help define the thermal unfolding of this tRNA. Clearly the study of activation energies measured by exchange of imino protons can be extremely useful in understanding the dynamics of nucleic acids in solution.

The study of the exchange rates of imino protons can be used in model nucleic acid systems to study the effect of a perturbation in a helix on the rest of the base pairs in the double strand. We have probed the effect and extent of several perturbations (a G·T base pair and an extra adenine base on one strand) on the lifetimes for opening of the other base pairs in the double strand (A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results).

Exchange rate measurements can also be used in studies of drug–nucleic acid interactions. Examples where this technique would be useful include studies that try to define the site, or sequence dependence, of drug binding as well as changes in the dynamics of base-pair opening due to drug binding. Such studies are underway in our laboratory and show promise as new probes of drug–nucleic acid interactions.

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Interaction of the *Hpa*I Endonuclease with Synthetic Oligonucleotides†

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ABSTRACT: To determine which functional groups of bases within the grooves of double-helical DNA interact with the *Hpa*I endonuclease, we have employed chemically synthesized octanucleotides containing base analogues. The 5-methyl group of thymine was probed as a contact between the *Hpa*I endonuclease and its recognition sequence by using the oligonucleotides d(G-G-T-T-A-A-C-C), d(G-G-T-U-A-A-C-C), and d(G-G-T-U(Br)-A-A-C-C). The 2-amino group of guanine was probed as a contact for the *Hpa*I endonuclease by using the octanucleotide d(G-I-T-T-A-A-C-C). The *Hpa*I endonuclease cleaves octanucleotides d(G-G-T-T-A-A-C-C) and d(G-G-T-B-A-A-C-C) according to Michaelis–Menten kinetics. However, both the K_m and turnover number for d(G-G-T-B-A-A-C-C) were severalfold lower than those for cleavage of d(G-G-T-T-A-A-C-C). In addition, d(G-G-T-U-A-A-C-C) was not cleaved by *Hpa*I endonuclease, suggesting that the 5-methyl group of thymine is a contact between the *Hpa*I endonuclease and its recognition sequence. d(G-I-T-T-A-A-C-C) was not cleaved by the *Hpa*I endonuclease which may be due in part to the low thermal stability of the duplex. Nevertheless, our results suggest that the 2-amino group of guanine is a contact for the *Hpa*I endonuclease. A phosphate group 5′ external to the *Hpa*I recognition sequence has been identified as a contact between the *Hpa*I endonuclease and DNA. The *Hpa*I endonuclease cleaved 5′-phosphorylated octanucleotide 30-fold faster than unphosphorylated octanucleotide. In addition, the K_m of the d(G-G-T-T-A-A-C-C) was 8000-fold higher than the K_m of the phage f_1 RFI DNA, suggesting that the octanucleotide is too short to take advantage of the entire DNA binding site of the enzyme.

The simplicity of structure and activity requirements has rendered type II endonucleases and their cognate methylases an attractive system for studying sequence-specific DNA–protein interactions (Modrich, 1979). Since the endonuclease and methylase are normally present as pairs in the cell, they provide an excellent opportunity to study the interactions of two entirely different enzymes with the same DNA sequence. Our laboratory has purified to homogeneity the type II endonuclease and methylase occurring in the bacterium *Haemophilus parainfluenzae*. The *Hpa*I endonuclease, *Hpa*II endonuclease, and their cognate methylases have been characterized with respect to molecular weight, oligomeric state, and kinetic parameters of the DNA cleavage and methylation reactions (Hines, 1979; Hines & Agarwal, 1979; Yoo & Agarwal, 1980; Yoo, 1981; Dwyer-Hallquist, 1981). In this paper, we report studies on the mechanism by which the *Hpa*I

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