in the same way. In addition, when biological effects are considered in vivo, other selective effects must be evaluated experimentally since it is well-known that reactions that appear to be kinetically inconsequential can produce amplifiable biological effects. While we make no claim that chain cleavage by 254-nm light is more important than pyrimidine dimer formation, or any other photoreaction, we do believe the reaction deserves further study both chemically and biologically.

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Kinetics for Exchange of Imino Protons in the d(C-G-C-G-A-A-T-T-C-G-C-G) Double Helix and in Two Similar Helices That Contain a G·T Base Pair, d(C-G-T-G-A-A-T-T-C-G-C-G), and an Extra Adenine, d(C-G-C-A-G-A-A-T-T-C-G-C-G)†

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ABSTRACT: The relaxation lifetimes of imino protons from individual base pairs were measured in (I) a perfect helix, d(C-G-C-G-A-A-T-T-C-G-C-G), (II) this helix with a G·C base pair replaced with a G·T base pair, d(C-G-T-G-A-A-T-T-C-G-C-G), and (III) the perfect helix with an extra adenine base in a mismatch, d(C-G-C-A-G-A-A-T-T-C-G-C-G). The lifetimes were measured by saturation recovery proton nuclear magnetic resonance experiments performed on the imino protons of these duplexes. The measured lifetimes of the imino protons were shown to correspond to chemical exchange lifetimes at higher temperatures and spin–lattice relaxation times at lower temperatures. Comparison of the lifetimes in these duplexes showed that the destabilizing effect of the G·T base pair in II affected the opening rate of only the nearest-neighbor base pairs. For helix III, the extra adenine affected the opening rates of all the base pairs in the helix and thus was a larger perturbation for opening of the base pairs than the G·T base pair. The temperature dependence of the exchange rates of the imino proton in the perfect helix gives values of 14–15 kcal/mol for activation energies of A·T imino protons. These relaxation rates were shown to correspond to exchange involving individual base pair opening in this helix, which means that one base-paired imino proton can exchange independent of the others. For the other two helices that contain perturbations, much larger activation energies for exchange of the imino protons were found, indicating that a cooperative transition involving exchange of at least several base pairs was the exchange mechanism of the imino protons. The effects of a perturbation in a helix on the exchange rates and the mechanisms for exchange of imino protons from oligonucleotide helices are discussed.

Relaxation rates of the base-paired imino protons have been measured by proton nuclear magnetic resonance (NMR) experiments in several nucleic acid systems (Crothers et al., 1974; Johnston & Redfield, 1977, 1978; Hurd & Reid, 1980; Early et al., 1981a,b). We recently studied the kinetics for exchange of imino protons in a DNA, RNA, and hybrid oligonucleotide helix (Pardi & Tinoco, 1982). The saturation recovery technique developed by Redfield (Johnston & Redfield, 1977) was used in these studies, and the theory for interpretation of the exchange behavior of imino protons measured by NMR has been discussed by Johnston & Redfield (1981) and Pardi & Tinoco (1982).

The three helices used in this work, the 12-mer [helix I = d(C-G-C-G-A-A-T-T-C-G-C-G)], the 12-mer G·T [helix II = d(C-G-T-G-A-A-T-T-C-G-C-G)], and the 13-mer [helix III = d(C-G-C-A-G-A-A-T-T-C-G-C-G)], have been studied

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by ^1H and ^{31}P NMR (Patel et al., 1982a–c). The conformation and dynamics of these duplexes were observed by measuring the chemical shifts and nuclear Overhauser effects on the imino, base, and sugar protons. These studies demonstrated the existence of a G·T wobble base pair in helix II and showed that the extra adenine base in helix III was stacked in the helix (Patel et al., 1982b,c). The destabilizing influence of a G·T base pair or an extra adenine is reflected in the melting temperatures of these helices, which are approximately 57 and 52 °C in 0.1 M phosphate for the 13-mer and 12-mer G·T helices, compared to the ~72 °C found for the 12-mer helix, under the same conditions. The NMR of these three helices, including preliminary reports of some of the work presented here, has recently been reviewed (Patel et al., 1982d).

In this paper we have measured the relaxation rates of the imino protons in the 12-mer, 12-mer G·T, and the 13-mer using saturation recovery experiments. Activation energies for exchange of the imino protons were determined by measuring the temperature dependence of the lifetimes. The pH dependence of the relaxation rates of the imino protons in the 12-mer and 12-mer G·T was also measured. The lifetimes, the pH dependence of the lifetimes, and the activation energies for exchange of the imino protons allow the dynamics of these duplexes to be interpreted in terms of specific mechanisms for exchange of the imino protons. The results on the 12-mer G·T and 13-mer show that helix opening is important in exchange of these imino protons. The helix opening pathway is shown to be the dominant exchange mechanism in the DNA duplex, $d(CA_5G) + d(CT_5G)$ (Pardi & Tinoco, 1982). For the 12-mer duplex the exchange of the imino protons takes place by an individual base pair opening mechanism. The effects of a G·T wobble base pair, and an extra adenine, on the opening rates of individual base pairs in the three helices are discussed, along with the general dynamics of base pair opening in double-helical oligonucleotides.

Materials and Methods

The oligonucleotides were prepared by a modified triester method followed by deprotection and purification (Hirose et al., 1978; Patel et al., 1982a–c). The NMR experiments were performed on the HXS-360-MHz instrument at the Stanford Magnetic Resonance Laboratory, with the experimental methods described previously (Pardi & Tinoco, 1982). The NMR spectra were all run in 0.1 M phosphate buffer–2.5 mM ethylenediaminetetraacetic acid (EDTA) with the chemical shifts referenced to the internal standard sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The lifetimes were calculated, as previously discussed (Pardi & Tinoco, 1982), with no significant double-exponential behavior seen in any of the data. The lifetimes reported here are estimated to be accurate to within ±20%. Typically, 10–15 different delay times were taken with 220–250 scans for each point.

Some of the experiments discussed here were repeated on a 200-MHz instrument using methods similar to the long-pulse inversion recovery technique described by Early et al. (1980). The values for the lifetimes calculated from these experiments are not reported here but were found to be within experimental error of the reported values.

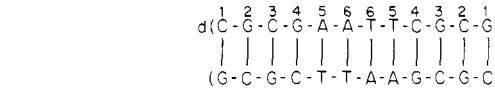
Results

12-mer: *d(C-G-C-G-A-A-T-T-C-G-C-G)*. Figure 1 shows a saturation recovery experiment performed on the 12-mer at 15 °C, pH 8. As discussed by Patel et al. (1982a), the terminal G·C base-paired imino proton was observed only at very low temperatures and so was not seen in the temperature range used in this study. The measured lifetimes of the other five imino resonances for the 12-mer at pH 6 are given as a function of temperature in Table I. A saturation recovery experiment on an interior A·T imino resonance (base pair 5) in the 12-mer is shown in Figure 2. Arrhenius plots for the lifetimes of base pairs 3–6 are shown in Figure 3.

The pH dependence of the lifetimes of the imino protons in the 12-mer was also measured to test for open-limited behavior in these protons. The measured lifetimes of the imino protons in the 12-mer are given at pH 6 and pH 8 for several temperatures in Table II.

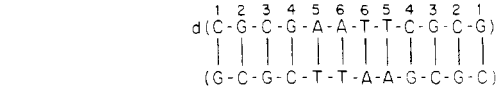
12-mer G·T: *d(C-G-T-G-A-A-T-T-C-G-C-G)*. Figure 4 shows an example of a saturation recovery experiment on the 12-mer G·T at 15 °C, pH 8. The measured lifetimes of the imino protons on base pairs 2–6 at pH 6 are given as a function

Table I: Lifetimes (ms) of Imino Protons in 12-mer at pH 6



temp (°C)	proton 2	3	4	5	6
5	150	260	230	250	270
15	140	205	255	360	320
25	100	140	190	310	350
30	90	175	240	230	280
35	40	130	170	140	235
40	30	80	160	125	200
45	18	60	150	90	130
50		50	80	65	90
55		35	80	35	55

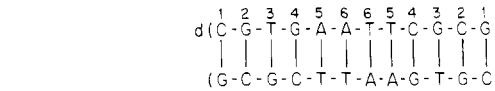
Table II: pH Dependence of Lifetimes (ms) of Imino Protons in 12-mer



pH	temp (°C)	proton 2	3	4	5	6
6	15	140	205	255	360	320
8		160	230	260	275	280
6	35	40	130	170	140	235
8		23	170	280	180	235
6	45	18	60	150	90	130
8		*a*	*b*	180	70	105

[a] Too fast to measure, <5 ms. [b] Lifetime is difficult to measure due to overlapping peaks at this temperature.

Table III: Lifetimes (ms) of Imino Protons in 12-mer G·T at pH 6



temp (°C)	proton 2	3 G	3 T	4	5	6
5		210	210	160	225	255
15		170	150			
20	40	90	110	185	200	260
25	30	55	55	150	235	250
30		35	35	90	205	255
35	9	12	12	85	135	225
40				40	75	95
45				15	20	19
50				9		

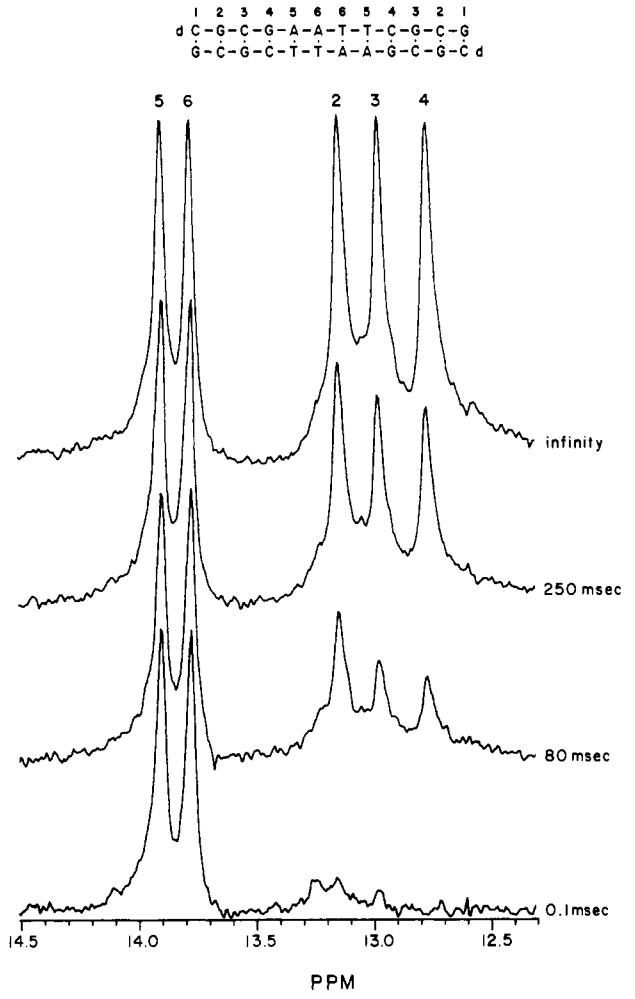


FIGURE 1: Spectra of a saturation recovery experiment (only partial data shown) on imino protons in the 12-mer double strand at 15 °C and pH 8. The peaks at 13.15, 12.98, and 12.78 ppm, corresponding to imino protons on base pairs 2, 3, and 4, respectively, were saturated. The times in milliseconds correspond to the delay times between saturation and the detection pulses. The spectrum at infinite time was taken under the same conditions with no power in the saturation pulse.

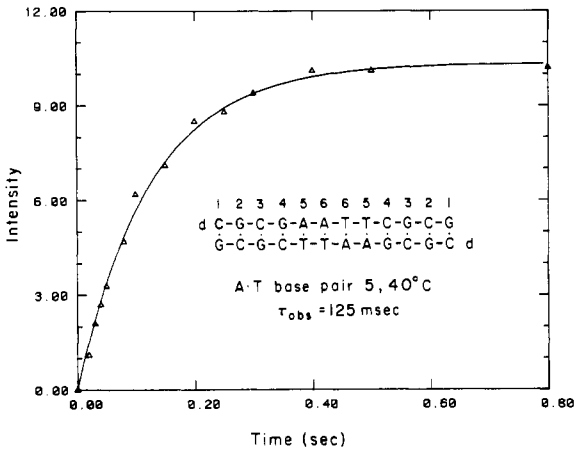


FIGURE 2: Saturation recovery experiment on an interior A·T imino resonance (base pair 5) in the 12-mer. The triangles are experimental data, and the solid line is the best fit of the lifetime, τ_{obsd}.

of temperature in Table III. Arrhenius plots for the lifetimes of base pairs 4–6 are shown in Figure 5. The pH dependence of the lifetimes of the imino protons in the 12-mer G·T for several temperatures is shown in Table IV.

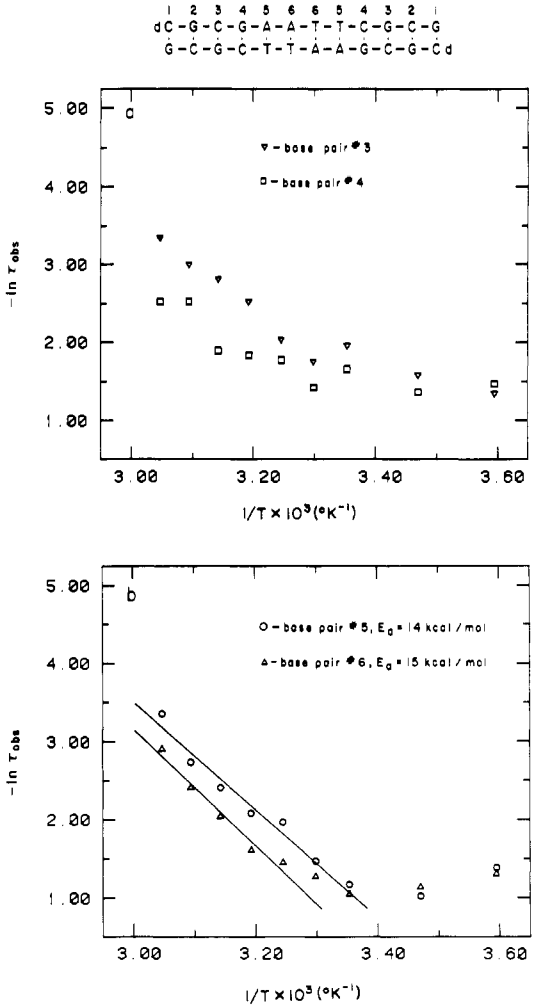


FIGURE 3: Arrhenius plots for observed lifetimes of the 12-mer double helix for (a) G·C base pairs and (b) A·T base pairs. The line used to determine the activation energies was calculated from the first five points (temperatures of 35 °C and above.)

Table IV: pH Dependence of Lifetimes (ms) of Imino Protons in 12-mer G·T

1 2 3 4 5 6 6 5 4 3 2 1
d(C-G-T-G-A-A-T-T-C-G-C-G)
(G-C-G-C-T-T-A-A-G-T-G-C)d

		proton					
			3				
pH	temp (°C)	2	G	T	4	5	6
6	20	40	90	110	190	200	260
8		20	32	30	220	260	270
6	30		35	35		200	250
8				10		200	260
6	40				40	75	95
8					60	85	130

13-mer: *d(C-G-C-A-G-A-A-T-T-C-G-C-G)*. Figure 6 shows a saturation recovery experiment on the 13-mer at 15 °C, pH 8. The measured lifetimes of the imino protons at pH 8 are given as a function of temperature in Table V. Arrhenius plots for the lifetimes of base pairs 4–6 are shown in Figure 7.

Discussion

T_1 vs. Chemical Exchange. By measuring the relaxation lifetimes while varying temperature and pH, we can begin to

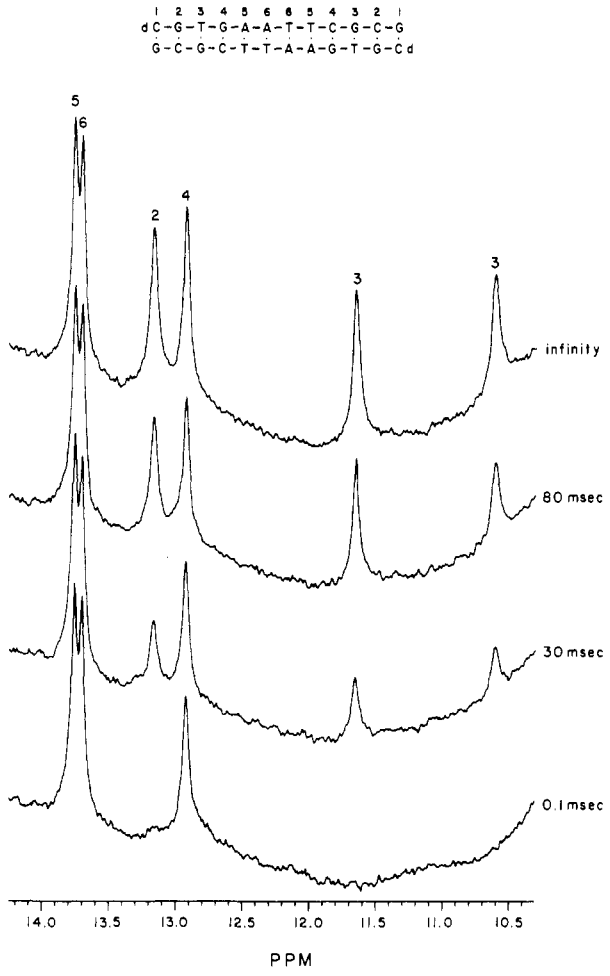
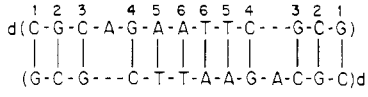


FIGURE 4: Spectra of a saturation recovery experiment (only partial data shown) on imino protons in the 12-mer G·T helix at 15 °C and pH 8. The peaks at 13.16, 11.65, and 10.6 ppm, corresponding to base pairs 2 and the imino protons from the G·T base pair, respectively, were saturated. Assignment of the low field resonance to the T and the high field resonance to the G in the G·T base pair was made by Patel et al. (1982c). The times in milliseconds correspond to the delay times between saturation and the detection pulses. The spectrum at infinite time was taken under the same conditions with no power in the saturation pulse. The curvature of the base line in these spectra is due to the use of Redfield 214 pulse.

Table V: Lifetimes (ms) of Imino Protons in 13-mer at pH 8



temp (°C)	proton 2	3	4	5	6
5		68	175	230	280
10			150	240	260
15	32	45	160	250	290
20			195	300	270
25			155	220	265
30			100	150	225
35			60	85	165
40			32	45	60
45			15	14	22

understand the mechanism for exchange. We will consider two processes that contribute to the relaxation lifetimes: chemical exchange and all other processes that do not involve exchange; these other processes will be called spin–lattice relaxation. At temperatures well below the melting temperature the lifetimes measured by NMR are dominated by the

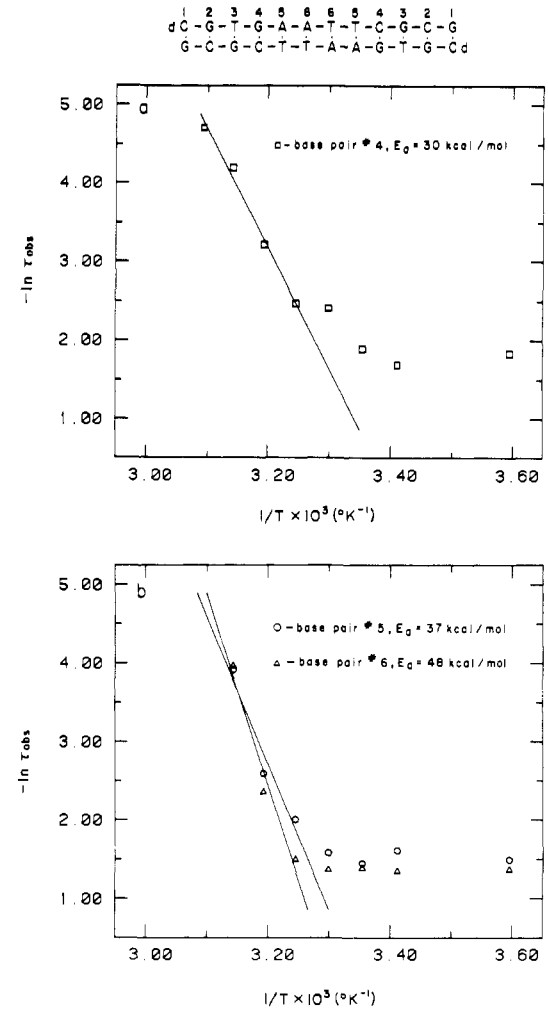


FIGURE 5: Arrhenius plots for observed lifetimes of the 12-mer G·T helix for (a) G·C base pairs and (b) A·T base pairs. The activation energy was calculated from the first four points in (a) and the first three points in (b) (temperatures of 35 °C and above).

spin–lattice relaxation time, T_1, of the imino protons. As the temperature approaches the melting temperature, or T_m, chemical exchange with water dominates the observed lifetimes (Johnston & Redfield, 1978; Early et al., 1981a,b; Pardi & Tinoco, 1982).

In the 12-mer the T_1 becomes important below 30 °C. This can be seen in Table I where the lifetimes of the imino protons increase sharply when the temperature changes from 55 to 25–30 °C. For base pairs 4–6, the lifetimes then level off until 5 °C where they decrease again. The contribution of T_1 at low temperatures becomes more apparent in the Arrhenius plots for the lifetimes of these protons (Figure 3). The dominant contribution to the observed lifetimes for temperatures above 30 °C is chemical exchange of the imino protons with water, which is consistent with studies on other systems (Johnston & Redfield, 1978; Early et al., 1981a,b; Pardi & Tinoco, 1982). Similar behavior can be seen for the imino protons on the 12-mer G·T and 13-mer. This is illustrated for the 12-mer G·T in Table III and Figure 5 and for the 13-mer in Table V and Figure 7. Therefore, we will concentrate on the lifetimes above 30 °C, which measure the chemical exchange of the imino protons in all three helices.

The lifetimes at the high temperatures will have some contribution due to the spin–lattice relaxation. A contribution from T_1 will result in a lifetime for chemical exchange that is greater than the observed lifetime. Because the low-temperature data, where T_1 is dominant, are limited to temper-

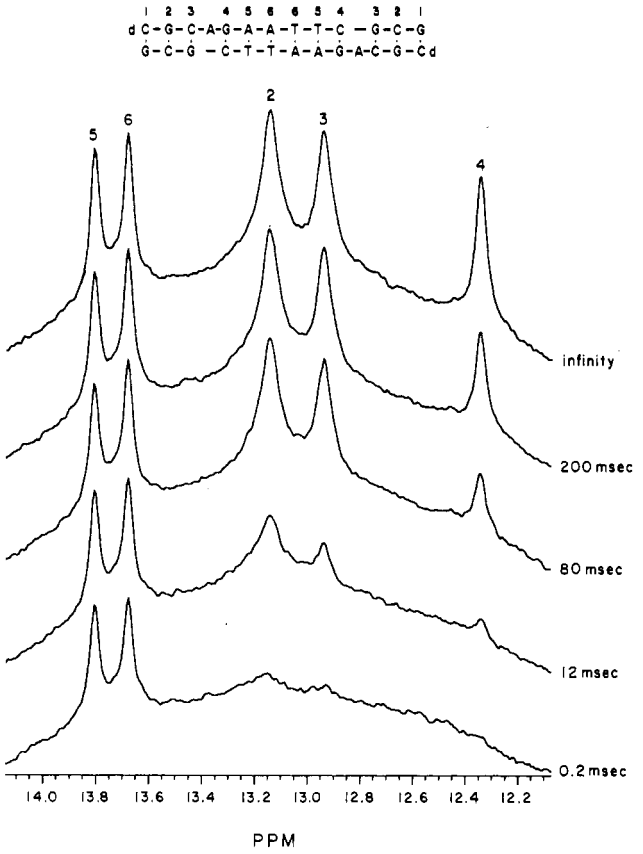


FIGURE 6: Spectra of a saturation recovery experiment (only partial data shown) on imino protons in the 13-mer helix at 15 °C and pH 8. The peaks at 13.14, 12.93, and 12.34 ppm, corresponding to base pairs 2, 3, and 4, respectively, were saturated. The times in milliseconds correspond to the delay times between saturation and the detection pulses. The spectrum at infinite time was taken under the same conditions with no power in the saturation pulse.

atures above 0 °C, it is difficult to determine the effect of T_1 accurately. However, we estimate that T_1 will increase the lifetimes for chemical exchange and the activation energies by not more than 50%. This will not change any of the qualitative conclusions we make in the following sections.

Are These Imino Protons in the Open-Limited Region for Exchange? The exchange rates are easily interpreted in terms of base pair opening rates if exchange is open limited. The exchange of the imino protons has been found to be in the open-limited region in tRNA (Hurd & Reid, 1980) and also for the interior base pairs in the double helix d(CA_5G) + d(CT_5G) (Pardi & Tinoco, 1982). To determine if exchange is open limited, we vary the concentration of the catalyst and observe any changes in the measured lifetimes. If the system is open limited, the lifetimes will be independent of the catalyst concentration. In these studies the catalysts are OH^- and phosphate, and the concentrations of these catalysts are changed by varying the pH.

The measured lifetimes for the imino protons in the 12-mer at pH 6 and pH 8 are given in Table II. The lifetimes should decrease as the concentration of base increases if the exchange is not in the open-limited region (Crothers et al., 1974; Hilbers, 1979; Pardi & Tinoco, 1982). At 35 and 45 °C only the imino proton from base pair 2 has a lifetime that shows this decrease on going from pH 6 to pH 8. The measured lifetimes for base pairs 3–6 above 30 °C therefore measure the rates for opening of these base pairs. The measured lifetimes for base pair 2 represent only an upper limit for the lifetime for base pair opening. This is the same result found for the terminal and penultimate base pairs in the double helix d(CA_5G) + d-

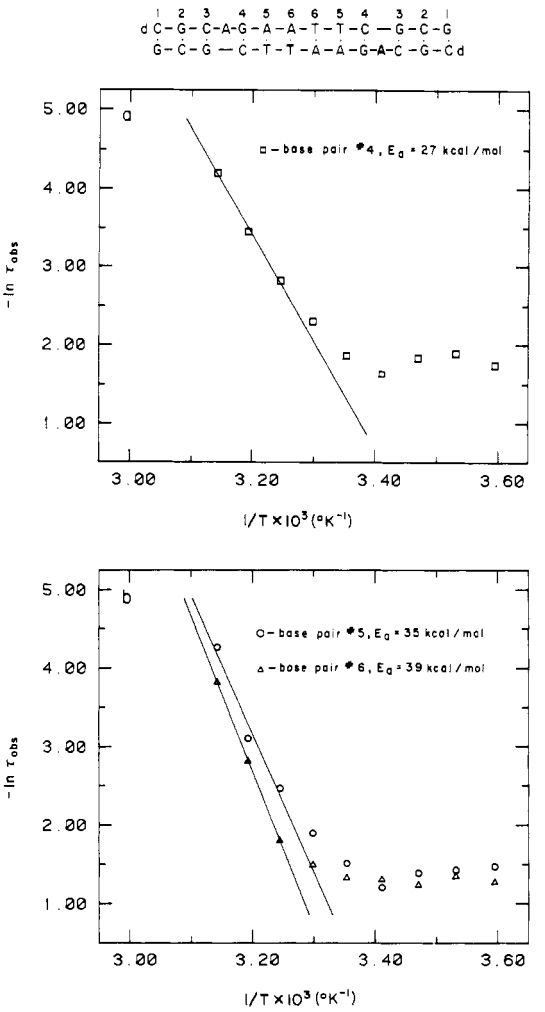


FIGURE 7: Arrhenius plots for observed lifetimes of the 13-mer helix for (a) G·C base pairs and (b) A·T base pairs. The activation energy was calculated from the first three points (temperatures of 35 °C and above).

(CT_5G) (Pardi & Tinoco, 1982). In the 12-mer G·T we again find chemical exchange to be predominant for the lifetimes above 30 °C in base pairs 4–6 (Table III). The pH dependence of the imino protons in the 12-mer G·T is given in Table IV. For base pairs 4–6, exchange is independent of pH and therefore is in the open-limited region. At 20 °C the lifetimes of the imino protons on base pairs 2 and 3 decrease by a factor of 2 or more on going from pH 6 to pH 8. The dependence of these protons on pH indicates that opening the base pair is not the rate-limiting step in the exchange of these imino protons.

In the 13-mer we will again consider only points above 30 °C and will thus only be measuring chemical exchange (Table V). The pH dependence of the lifetimes of the 13-mer was not measured. We will assume that base pairs 4–6 are analogous to those base pairs in the 12-mer and 12-mer G·T and thus are in the open-limited region.

Effects of Perturbations on Lifetimes of Exchange. By comparing relaxation lifetimes of our three oligonucleotides, we can determine the effects of a G·T base pair or an extra nucleotide on a double-stranded helix. For the 12-mer at 35 °C and pH 6, the lifetimes for base pairs 4, 5, and 6 are 170, 140, and 235 ms, respectively (Table I).

In the 12-mer G·T at 35 °C and pH 6 the lifetimes are 85, 135, and 225 ms for base pairs 4, 5, and 6, respectively (Table III). Lifetimes of base pairs 5 and 6 are comparable to those in the 12-mer, but base pair 4, which is next to the G·T base

Table VI: Activation Energies (kcal/mol) for Exchange of Imino Protons

molecule	proton 4	5	6
12-mer		14 ± 2	15 ± 2 (pH 6)
12-mer G·T	30 ± 5	37 ± 8	48 ± 9 (pH 6)
13-mer	27 ± 5	35 ± 8	39 ± 8 (pH 8)

pair, has decreased by a factor of 2. Although the G·T containing helix is destabilized relative to the 12-mer (a decrease in T_m of ~20 °C), these lifetimes show that the perturbation does not affect the lifetimes of the interior A·T base pairs and therefore is a very local effect. The lifetimes of both the G and T imino protons in the G·T base pair are the same at all temperatures, which means that when this base pair opens, both protons exchange at the same rate. At 35 °C the G·T base pair (3) has a lifetime of 12 ms compared to 130 ms for the analogous G·C base pair in the 12-mer.

For the 13-mer at 35 °C and pH 8, lifetimes for base pairs 4, 5, and 6 are 60, 85, and 165 ms, respectively (Table V), compared to 280, 180, and 235 msec in the 12-mer at pH 8 (Table II). Thus all of the lifetimes have decreased in the 13-mer. The perturbation due to the extra adenine is not localized as in the G·T case but affects the whole molecule.

Activation Energies and Exchange Mechanisms. By investigating the temperature dependence of the exchange rates of the imino protons, it is possible to obtain activation energies for the exchange process. The magnitudes of these activation energies can give information on the mechanisms by which exchange takes place (Pardi & Tinoco, 1982).

The activation energies for exchange of the imino protons of base pairs 5 and 6 of the 12-mer are shown in Table VI and were calculated from the plots in Figure 3. Points from 35 to 55 °C were used to calculate these activation energies. As discussed in a previous paper (Pardi & Tinoco, 1982), there may be several mechanisms for chemical exchange of the imino protons in these molecules, including single base pair opening and whole helix opening. We would expect the activation energy for helix opening of a duplex the size of the 12-mer to be approximately the enthalpy for double-strand formation, 102 kcal/mol, as determined by calorimetry (Patel et al., 1982a). The values measured for the exchange of the imino protons in the 12-mer are 14–15 kcal/mol. These low values indicate that helix opening is not a dominant process in the exchange of these protons and suggest that single base pair opening may be the important pathway for exchange. Other evidence for single base pair opening comes from the lifetimes of the individual base pairs. At 45 °C exchange lifetimes of base pairs 4, 5, and 6 are 150, 90, and 130 ms, respectively (Table I). Because the lifetime of base pair 5 is much shorter than either of its neighbors, this imino proton must, to some extent, exchange independently of the other two. Our results are in agreement with Early et al. (1981a,b), who found activation energies of around 15 kcal/mol for the A·T imino protons in another dodecamer. The difference in lifetimes (or rates) of the two A·T base pairs could be due to small differences in their activation energies or differences in the preexponential factor, A, for each base pair as given in the Arrhenius equation:

$$k = Ae^{-\Delta E_a/(RT)} \quad (1)$$

A difference in the activation entropies for the two A·T imino protons in the 12-mer might reflect differences in the flexibility of the base pairs. The activation energies for base pairs 3 and 4 were not calculated because it is not clear where chemical exchange becomes the dominant mechanism for the relaxation lifetimes.

For the 12-mer G·T, the imino protons broaden and disappear at a lower temperature than in the 12-mer. Therefore, we could only take measurements at three or four temperatures above 30 °C to use for calculation of the activation energies, and thus these values are less reliable than the values obtained for the 12-mer. Table VI gives the activation energies for the 12-mer G·T. The Arrhenius plot is shown in Figure 5. It is clear that the activation energies for exchange of the imino protons in the 12-mer G·T are much larger than those in the 12-mer. This indicates single base pair opening is no longer the dominant pathway for exchange in the 12-mer G·T and that helix opening is probably contributing to the exchange. The activation energies for base pairs 4, 5, and 6 in the 12-mer G·T are 30, 37, and 48 kcal/mol, respectively (Table VI). If exchange were taking place by helix opening, we would expect the activation energies and lifetimes to be the same for all the imino protons. This result was found in the heptamer d-(CA_5G) + d(CT_5G) (Pardi & Tinoco, 1982). Thus the measured activation energies for the 12-mer G·T are probably due to contributions from both base pair opening and helix opening.

The activation energies for the imino protons of base pairs 4–6 of the 13-mer are given in Table VI and were calculated from the Arrhenius plots in Figure 7. Again only points above 30 °C were used. The activation energies for base pairs 4, 5, and 6 are 27, 35, and 39 kcal/mol, respectively. This is very similar to the 12-mer G·T in that the high activation energies probably indicate contributions from both base pair opening and helix opening.

In order to better understand how contributions from both base pair opening and helix opening will affect the rates of exchange and the activation energies, we have calculated rates for the system at different temperatures using the model developed in a previous paper (Pardi & Tinoco, 1982). For a system where two pathways contribute to the exchange of an imino proton, the observed activation energy, ΔE_{obsd}, is given by

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

In this equation k_{op}^{I} and k_{op}^{II} are the rate constants and ΔE_{op}^{I} and ΔE_{op}^{II} are the activation energies for (I) the base pair opening and (II) the helix opening processes, respectively. We can obtain values for ΔE_{op}^{II} and k_{op}^{II} by measuring the kinetics of the helix–coil transition using temperature–jump techniques (Pörschke & Eigen, 1971). Values were measured for the 12-mer G·T and the 13-mer in the same buffer as the NMR experiments. The activation energies for dissociation of the double helix (ΔE^{II}) were 68 and 74 kcal/mol for the 12-mer G·T and 13-mer, respectively. The preexponential factor, A, for this process was found to be 1.2×10^{48} and 2.6×10^{52} s^{-1} for the 12-mer G·T and 13-mer, respectively (Y. G. Chu and I. Tinoco, Jr., unpublished results). We used the data from base pair 6 of the 12-mer to get approximate values for base pair opening: ΔE_{op}^{I} = 14 kcal/mol; $A = 8 \times 10^{10}$ s^{-1}. Using these numbers and the Arrhenius equation (eq 1), we can calculate the observed rate constant for this two-pathway process at any temperature. The observed rate will be the sum of the individual rates for each pathway. Figure 8 shows an Arrhenius plot calculated for the 12-mer G·T with this model. At high temperature the steeper slope indicates a larger activation energy (68 kcal/mol), which is the activation energy for helix opening. At lower temperature base pair opening

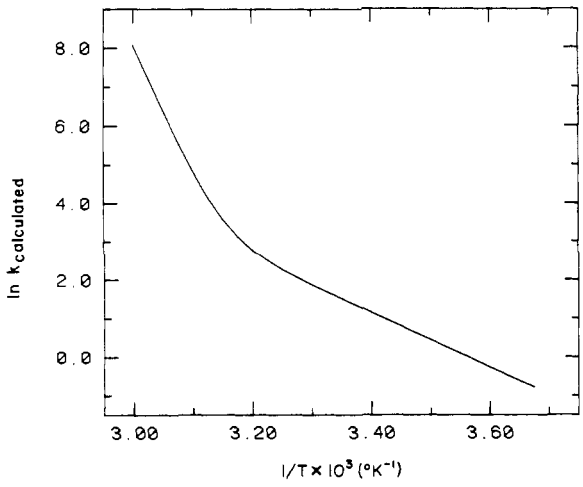


FIGURE 8: An Arrhenius plot calculated for the no. 6 A·T base pair of the 12-mer G·T helix, assuming only helix opening and base pair opening for exchange of the imino proton. Discussion of these exchange mechanisms and values for the rates are given in the text.

becomes dominant, and we see a smaller activation energy (14 kcal/mol). This model predicts that the helix opening pathway becomes dominant above 40 °C, that below 30 °C base pair opening is the main exchange pathway, and between 30 and 40 °C both pathways are important. This is consistent with our interpretation of the measured activation energies for the 12-mer G·T and the 13-mer. It is clear from these results that NMR experiments alone will not always give enough information to explain the kinetics of exchange in oligonucleotides and may actually be misleading by themselves. Thus complementing the NMR kinetic measurements with temperature–jump kinetics will lead to a much better understanding of the dynamics of the helices in solution.

Conclusions

This paper describes experiments that probe the relaxation lifetimes of imino protons of base pairs in different environments. The effects of destabilizing perturbations in a helix on the exchange rates have been studied. The saturation recovery NMR experiments performed here allow the lifetimes for opening of individual base pairs in a helix to be determined. We were thus able to study the kinetic stability of each base pair, instead of just the overall stability of the whole helix. The three molecules used in this paper, the 12-mer d(C-G-C-G-A-A-T-T-C-G-C-G), the 12-mer G·T d(C-G-T-G-A-A-T-T-C-G-C-G), and the 13-mer d(C-G-C-A-G-A-A-T-T-C-G-C-G), represent excellent models for probing the effects of a perturbation (a G·T base pair and an unpaired adenine) on the stability of individual base pairs in the helix. Patel et al. (1982a–c) have reported on the temperature dependence of the chemical shifts of the imino protons in these molecules and obtained information on the conformations and the relative stabilities of these molecules.

The observed lifetimes measured in the three helices studied in this paper were shown to correspond to lifetimes for chemical exchange of the imino protons for temperatures above 30 °C. These lifetimes were also shown to be in the open-limited region (every time the base pair opens the imino proton exchanges with solvent water) for base pairs 3–6 in the 12-mer and for base pairs 4–6 in the 12-mer G·T and the 13-mer.

Comparison of the lifetimes in these helices shows that the G·T base pair in the 12-mer G·T causes an order of magnitude decrease in the relaxation lifetime of its imino protons relative to the G·C base pair in the 12-mer. The destabilization of the G·T base pair also has a large effect on the neighboring base pair (4) but does not affect the lifetime of the next nearest-neighbor A·T base pair in the helix. Thus the effect of the G·T base pair is very localized in the helix. The destabilization due to the mispaired adenine in the 13-mer has a larger effect on the helix, with the lifetimes of all the imino protons decreasing, relative to the 12-mer.

Activation energies for exchange of the imino protons were obtained by observation of the temperature dependencies of the exchange rates. In the 12-mer both the A·T imino protons have activation energies of 14–15 kcal/mol. The values were shown to correspond to exchange of the imino protons by an individual base pair opening mechanism. Early et al. (1981b) found the same results for the activation energy for exchange of A·T imino protons in other oligonucleotide helices. The activation energies for the imino protons in the 12-mer G·T and 13-mer are much larger in magnitude than those in the 12-mer, indicating a helix opening mechanism is involved in the exchange of the imino protons in these two helices. The helix opening pathway was shown to be the dominant exchange process of the double-strand $d(CA_5G) + d(CT_5G)$ studied in a previous paper (Pardi & Tinoco, 1982).

It is clear from these studies that the relaxation lifetimes of an imino proton in a double helix are dependent upon many factors. Whether the imino proton is in an A·T or G·C base pair will affect its lifetime, as will the sequence of its neighboring base pairs. A very important factor in the exchange of an imino proton in a double helix is its distance from a terminal base pair. Terminal base pairs are known to kinetically fray (Patel & Hilbers, 1975; Pardi & Tinoco, 1982), which means that they open and close at very fast rates. This kinetic fraying of the ends of a helix will affect the rates of exchange for the penultimate base pairs on ends of a helix and possibly the third base pair in from the end, depending upon the sequence and the temperature. A perturbation in a helix will also have a large effect upon the exchange lifetimes of the imino protons in a double helix. These perturbations could be destabilizing factors such as internal loops, bulges, or nonstandard base pairs such as the G·T base pair studied in this work. Many drugs are known to bind to nucleic acids and to stabilize the double helix; these drugs would be expected to have a large effect on the exchange rates of the imino protons in the helix [see Patel et al. (1982d) for a preliminary report of such studies]. Knowledge of the lifetime for exchange of imino protons of individual base pairs in a helix will give extremely valuable information on the extent of such perturbations throughout the helix.

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Identification and Quantitation of a 2.0-Kilobase Messenger Ribonucleic Acid Coding for 3-Methylcholanthrene-Induced Cytochrome P-450 Using Cloned Cytochrome P-450 Complementary Deoxyribonucleic Acid†

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ABSTRACT: We have used a plasmid containing DNA complementary to one of the two size classes of mRNA coding for 3-methylcholanthrene-induced cytochrome P-450 from rat liver to characterize and quantitate that mRNA. The plasmid used was constructed and identified as follows: Total poly(A^+) RNA from 3-methylcholanthrene-induced liver was used as a template for cDNA synthesis. Double-stranded cDNA was inserted into plasmid pBR322 by the G-C tailing procedure. Recombinants were screened by colony hybridization using as probe [^{32}P]cDNA prepared from partially purified cytochrome P-450 mRNA. A differential screening approach was used in which duplicate filters were hybridized with probe from either 3-methylcholanthrene treated or untreated rats. Plasmid p23 was strongly positive by colony hybridization. It was conclusively shown to contain cytochrome P-450 cDNA sequences by demonstrating that the mRNA which specifically hybridized to nitrocellulose-bound plasmid p23 could be translated in vitro into peptides that were immunoprecipitable with monoclonal antibodies specific for 3-methylcholanthrene-induced cytochrome P-450. The size and quantity of the mRNA complementary to plasmid p23 were determined by hybridization of the ^{32}P-labeled plasmid to rat liver RNA that had been fractionated by electrophoresis under fully denaturing conditions and transferred to diazobenzyloxymethyl-paper. Plasmid p23 hybridized strictly to a single size of mRNA that was about 2000 nucleotides in length, the smaller of the two size classes of mRNA coding for 3-methylcholanthrene-induced cytochrome P-450. From this we concluded that, at least within the region of the mRNA contained within the insert of plasmid p23, the two size classes of 3-methylcholanthrene-induced cytochrome P-450 mRNA were very different in sequence. The mRNA complementary to plasmid p23 was increased about 4-fold after in vivo administration of 3-methylcholanthrene under conditions that result in maximal induction of 3-methylcholanthrene-induced cytochrome P-450 enzymatic activity. This increase in cytochrome P-450 mRNA parallels the increase in cytochrome P-450 enzymatic activity observed after 3-methylcholanthrene administration. These data suggest that the regulation of mRNA levels is an important point of control of cytochrome P-450 gene expression.

The cytochromes P-450, the terminal enzymes of microsomal mixed-function oxidases, are central to the metabolism and enzymatic detoxification of a wide variety of endogenous metabolites and xenobiotics that include carcinogens, drugs, and steroids (Conney, 1967). This system is also responsible for the enzymatic activation of many compounds into mutagenic or carcinogenic forms (Gelboin, 1980). It is well established that there are multiple forms of the cytochromes P-450, many of which are regulated by a variety of inducers (Lu & West, 1980; Haugen et al., 1975; Guengerich & Mason, 1979).

Some of the mechanisms of induction of cytochrome P-450 enzymatic activity have been elucidated in cell culture (Nebert & Gelboin, 1968, 1970; Whitlock & Gelboin, 1974). Induction is caused by a variety of treatments including exposure to polycyclic aromatic hydrocarbons such as benzo[*a*]anthracene (Nebert & Gelboin, 1970; Whitlock & Gelboin, 1974) and exposure to cAMP (Yamasaki et al., 1975). Induction also occurs following temporary inhibition of protein synthesis (Whitlock & Gelboin, 1974). Both protein and RNA syntheses are required for induction (Nebert & Gelboin, 1968, 1970; Whitlock & Gelboin, 1970).

The molecular biology of the cytochromes P-450 and their regulation are presently under vigorous investigation. Recombinant DNA techniques are being used to develop the tools needed to examine cytochrome P-450 regulation at the levels of mRNA transcription, processing, and degradation and to elucidate the structure and multiplicity of the cytochrome P-450 genes. To date, cDNAs derived from three different cytochrome P-450 mRNAs have been cloned (Negishi et al., 1981; Fujii-Kuriyama et al., 1981; Bresnick et al., 1981; Adesnik et al., 1981). One cloned cDNA complementary to

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