Chemical Consequences of Incorporation of 5-Fluorouracil into DNA As Studied by NMR†

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ABSTRACT: The cytotoxic analogue of thymine, 5-fluorouracil (U^f), is known to be incorporated into DNA in biological systems. This abnormal base has been synthetically incorporated into short DNA oligomers. The ionization of the N-3 proton of this base within DNA oligomers was measured by observation of the ^{19}F chemical shift at varying pH values. The pK_a values for the U^f ring of dTpdU^fpdT and dApdU^fpdA were determined to be 7.84 and 7.9, respectively. The self-complementary 12-mers d(G-C-G-C-A-A-T-U^f-G-C-G-C) and d(C-G-A-T-U^f-A-T-A-A-T-C-G) were synthesized, and ^1H NMR was used to compare the helix dynamics and stability of the interstrand imino proton hydrogen bonds with those of the 12-mers d(G-C-G-C-A-A-T-T-G-C-G-C) and d(C-G-A-T-T-A-T-A-A-T-C-G). The N-3 hydrogen bond of the A-U^f base pair was less stable than the corresponding hydrogen bond in A-T base pairs in the same helix, and the A-U^f base pair was less stable than the A-T base pair in the analogous position of the control helix. The observed temperature-dependent dynamics and NMR melting temperatures of the control and dU^f-containing oligomers were similar.

5-Fluorouracil (U^f)[1] is a cytotoxic analogue of the natural base thymine and has proven useful in the chemotherapy of a number of cancers. The mechanism underlying its cytotoxicity is controversial. In addition to the inhibition of thymidylate synthase, there is evidence that cytotoxiciity is related to the incorporation of the drug into DNA and RNA (Wilkinson & Crumley, 1977; Kufe et al., 1981; Major et al., 1982; Schuetz et al., 1984). It has been hypothesized that the presence of the electron-withdrawing fluorine atom on the pyrimidine ring could result in the presence of ionized U^f in DNA or RNA at physiological pH. This could lead to mispairing with guanine during replication or transcription (Freese, 1959). The N-3 position of the nucleoside dU^f in solution is much more acidic than that of thymidine (Wempen & Fox, 1964; Cushley et al., 1968), and this relationship is maintained in the ribopolynucleotide poly(U^f) (Massoulie et al., 1963; Szer & Shugar, 1966).

The incorporation of U^f in place of thymidine in DNA may have other structural or functional ramifications that have not yet been explored. NMR spectroscopy has proven useful in examining the structural dynamics of DNA oligomers (Kan et al., 1982; Patel et al., 1982, 1985; Feigon et al., 1983; Mirau & Kearns, 1984, 1985). Parameters such as proton exchange with solvent, which serves as an indicator of base pair stability, can be determined with this technique (Fritzsche et al., 1983; Pardi et al., 1982). The change in chemical shift of imino protons with temperature has also been used as a probe of helix unwinding (Patel et al., 1982, 1985). The DNA oligomers "A-U^f 12-mer" and "Pribnow U^f" (Figure 1) were synthesized, along with both corresponding control 12-mers containing T in place of U^f. The stability of the overall helices was compared by studying the line broadening of the imino proton signals with respect to changes in temperature and buffer concentration for the U^f-containing and control oligomers. The change in chemical shift of the imino protons was also used as an indicator of premelting transitions occurring in the duplexes.

Materials and Methods

Materials. 5-Fluorodeoxyuridine was purchased from Sigma. 4,4′-Dimethoxytrityl chloride, protected nucleoside phosphoramidites, and other reagents for the solid-state phosphite triester synthesis were from Chem Genes Corp. (Needham, MA). All other chemicals were of reagent grade.

Methods. UV absorption spectra were measured on an LKB Ultrospec 4050. HPLC analysis was carried out on a Varian 500 HPLC equipped with an LKB 2140 rapid spectral detector, and interfaced with an IBM PC using Wavescan software from LKB. ^{19}F NMR spectra were obtained on a Bruker WM-300 FT-NMR at the Harvard University Department of Chemistry and were referenced to external trifluoroacetic acid, 10 mM, in D_2O. Proton NMR spectra were obtained by using a Bruker 500-MHz FT-NMR at the Yale University Chemical Instrumentation Center. These samples were studied in 90% H_2O/10% D_2O, and the water signal was diminished by the use of a 45–t–45 pulse sequence (Kime & Moore 1983). These spectra were referenced internally to the EDTA peak at 3.58 ppm. All samples were lyophilized prior to use and then dissolved in the appropriate buffer. EDTA was added to all buffers to chelate adventitious paramagnetic metal ions. In the buffer catalysis studies, t_{ex} was calculated from the relationship (Crothers et al., 1974)

$$t_{ex} = 1/\pi\Delta$$

where Δ is the difference between the line width at any buffer concentration and the line width of the nonexchanging proton in the absence of buffer. The latter value was estimated from the narrowest imino proton resonances in the samples that did not contain buffer.

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[1] Abbreviations: G, guanine; dG, deoxyguanosine; A, adenine; dA, deoxyadenosine; T, thymine; dT, thymidine; C, cytosine; dC, deoxycytosine; U^f, 5-fluorouracil; dU^f, 5-fluorodeoxyuridine; poly(U^f), poly(5-fluorouracil ribonucleotide); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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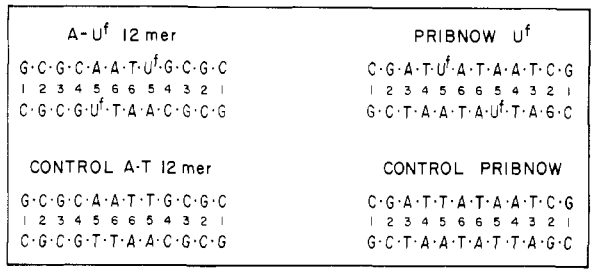

FIGURE 1: DNA oligomers used in this study.

Synthesis of Oligomers Containing U^f. 5-Fluorodeoxyuridine (1.84 g) was dissolved in dry pyridine, and the solution was evaporated to dryness in vacuo 3 times to render it anhydrous. The dry solid was then dissolved in 30 mL of dry pyridine, and 1.2 equiv of 4,4-dimethoxytrityl chloride was added in a single portion. The solution was stirred overnight at room temperature and the reaction terminated by pouring the solution into 100 mL of ice water. The mixture was extracted with $CHCl_3$, and the combined organic layer was washed successively with saturated NaCl, H_2O, and saturated $NaHCO_3$. The $CHCl_3$ was removed in vacuo, and the remaining pyridine was removed by coevaporation with toluene. The gummy yellow-brown product was purified by chromatography on silica gel, eluted with $CHCl_3$ and increasing concentrations of EtOH. Evaporation of the solvent gave 2.1 g of a yellow-white solid. The material was homogeneous by TLC on silica developed with $CHCl_3$/MeOH (9:1), $R_f = 0.41$.

This material was converted to the corresponding 3′-phosphoramidite with chloromethoxy-*N*,*N*′-diisopropylaminophosphine (Beaucage & Caruthers, 1981). This material was homogeneous by TLC on silica developed in $CHCl_3$ (45%), ethyl acetate (45%), and triethylamine (10%). The product was used in the solid-state phosphite triester oligonucleotide synthesis (Beaucage & Caruthers, 1981). For a typical trimer synthesis, 300 mg of silica-bound dimethyoxytrityl-dT, 60 μmol/mg, was reacted with 350 mg of the protected U^f phosphoramidite. The orange color following the subsequent removal of the dimethoxytrityl protecting group with $ZnBr_2$ indicated successful addition. Dimethoxytrityl-protected dT phosphoramidite, 400 mg, was then added to the bound dimer. The resultant trimer was deprotected, and removed from the solid support, with NH_4OH at 37 °C overnight. Removal of the solvent in vacuo yielded 10.7 mg of dTpdU^{f}pdT, as determined by measurement of the absorbance at 260 nm. The trimer was purified by HPLC on a C_{18} column using an acetonitrile gradient, O–20%, in 0.1 M triethylammonium bicarbonate, pH 7.5. Digestion of the product with snake venom phosphodiesterase yielded the appropriate monomers, as judged by HPLC analysis.

The 12-mers used in this study were synthesized by using an Applied Biosystems automated DNA synthesizer in the Yale University Department of Chemistry. The oligomer was cleaved from the solid support, and deprotected, with NH_4OH at 55 °C overnight. Purity was determined by reaction with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Maxam & Gilbert 1980), followed by gel electrophoresis.

RESULTS

Acidity of the N-3 Position of the U^f Ring. The ^{19}F NMR spectrum of 5-fluorodeoxyuridine, 3 mM in 100 mM phosphate buffer containing 5 mM EDTA, shows a shift of the position of the resonance from 90.3 ppm in the protonated state to 87.8 ppm when unprotonated. Measurement of the chemical shift as a function of pH yields a titration curve, with pK_a of 7.68 (Figure 2). This compares well with a pK_a of 7.58

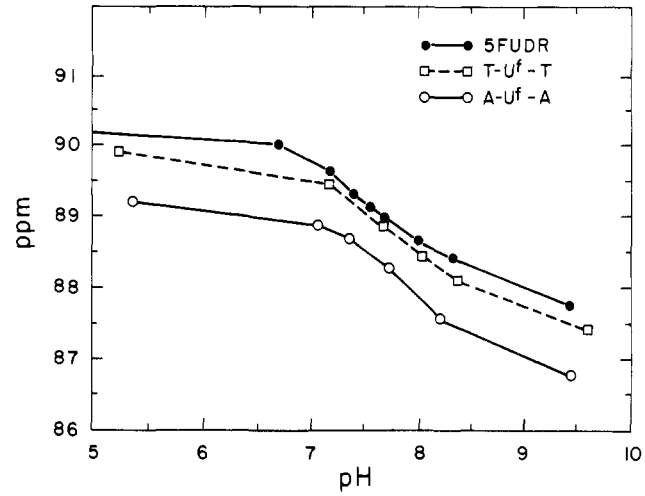

FIGURE 2: Titration curves for U^f-containing oligomers. All samples were 1 mM in 100 mM phosphate and 5 mM EDTA. Probe temperature was 298 K. (●) 5-FUDR; (□) dTpdU^{f}pdT; (○) dApdU^{f}pdA.

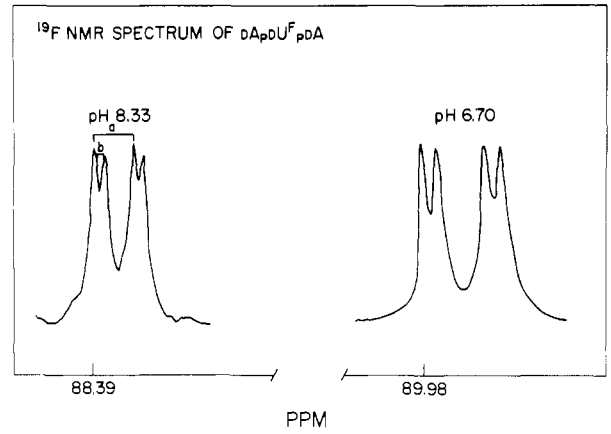

FIGURE 3: ^{19}F spectrum of dApdU^{f}pdA. Sample was 1 mM in 100 mM phosphate and 5 mM EDTA. (a) J_{F-H6}; (b) $J_{F-H1'}$.

obtained by measuring the change in UV absorbance of 5-fluorodeoxyuridine, 0.81 mM in 100 mM phosphate, as the ring is deprotonated. Values of 7.66, 7.98, and 8.00 have been reported in the literature without experimental details (Wempen & Fox, 1964; Cushley et al., 1968). The pK_a of the N-3 position of uridine derivatives is quite sensitive to temperature and ionic strength (Aylward, 1967). Presumably, variation in the solution conditions accounts for the variation in the reported pK_a. The ^{19}F spectrum of 5-fluorodeoxyuridine is comprised of a doublet of doublets (Cushley et al., 1968), with the major splitting due to coupling with H-6 and the minor splitting arising from long-range coupling to H-1′. These coupling constants are also pH dependent, with J_{F-H6} varying from 6.7 to 6.1 Hz as the molecule is deprotonated. Changes in the long-range coupling constant were too small to be accurately measured.

The ^{19}F spectra of the trimers dTpdU^{f}pdT and dApdU^{f}pdA, 1 mM in 100 mM phosphate and 5 mM EDTA, also show a doublet of doublets pattern (Figure 3). Both splittings were well resolved in the protonated and unprotonated states but were poorly resolved near the pK_a. The change in the ^{19}F chemical shift with pH for these trimers, as compared to 5-fluorodeoxyuridine, is shown in Figure 2. From the graph, it is possible to assign a pK_a of 7.84 to the N-3 position of dApdU^{f}pdA and a pK_a of 7.90 to that of dTpdU^{f}pdT. Incorporation of the U^f ring into an oligonucleotide, therefore, results in only a slight increase in the observed pK_a of the N-3

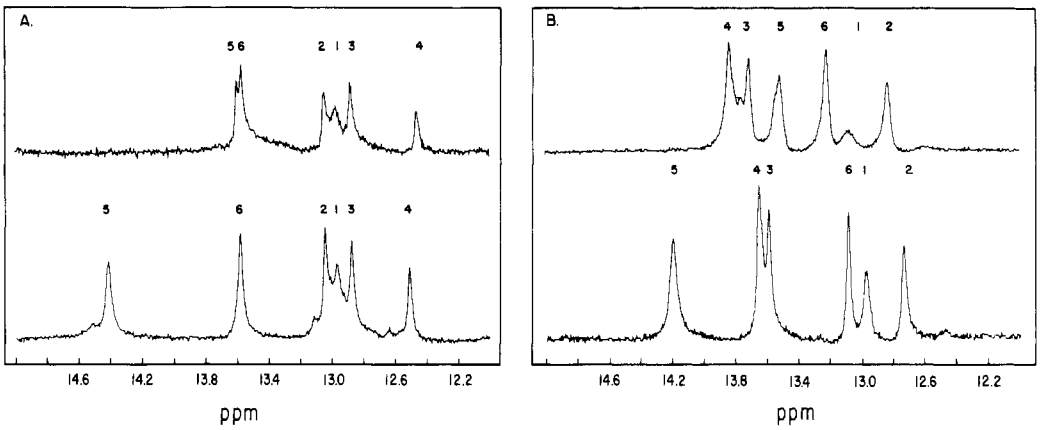

FIGURE 4: Proton NMR spectra of the imino proton region of U^f-containing 12-mers. (A) A-U^f 12-mer; 80 ODU/mL in 1 mM cacodylate, 100 mM NaCl, and 1 mM EDTA, pH 7.3 at 273 K. The control 12-mer is shown on top. At this temperature, the two A-T resonances of the control sample overlap. (B) Prib U^f 12-mer; 250 ODU/mL in 1 mM cacodylate, 100 mM NaCl, and 1 mM EDTA, pH 7.3 at 270 K. The control 12-mer is shown on top. Peak assignments are shown on the spectra.

position. The difference in pK_a between the two trimers is not significant, demonstrating that the difference between A and T in stacking interactions does not affect the pK_a of the U^f ring. Therefore, the N-3 position of the U^f ring is more acidic than the corresponding position in thymine, and a significant percentage of these molecules will be ionized at pH values in the physiological range.

Proton NMR Spectra. The NMR spectra of the hydrogen-bonded imino protons of the A-U^f and Pribnow U^f 12-mers are shown in Figure 4. The hydrogen-bonded imino protons of DNA base pairs are known to resonate between 12 and 15 ppm (Kearns et al., 1971). At low temperature, this region of the spectrum shows the six single lines expected for the six distinct base pairs. Assignments were made both by comparison of these spectra with the spectra of the control oligomers, as determined by us under the same conditions, and by reference to the published assignments for the control oligomers determined in somewhat different buffers (Patel et al., 1982a, 1983a, 1985), as well as the observed sequential fraying (Patel, 1974; Patel & Hilbers, 1975; Patel et al., 1982a; Kan, 1975). These considerations permitted assignment of the five natural base pairs; the remaining downfield peak was assigned to the A-U^f base pair by exclusion.

Premelting Transition. The effect of increasing temperature on the chemical shift of the imino protons of the U^f-containing oligomers is shown in Figure 5. The imino proton resonances of both oligomers show a definite upfield shift as the temperature increases. The shifts noted for the A-U^f base pairs are similar to those of the A-T base pairs over the temperature range in which the A-U^f signal can be observed. Of note, the temperature-dependent changes of these helices are virtually identical with those observed for the control helices, both in this experiment and in the literature (Patel et al., 1982, 1985).

Temperature-Induced Line Broadening. The effect of temperature on the line width of the imino protons of the U^f-containing helices is shown in Figure 6. These samples were dissolved in 1 mM cacodylate, 100 mM NaCl, and 1 mM EDTA, to avoid the contribution of buffer to line broadening. The signal of the A-U^f base pair is quite broad in both oligomers, even at low temperature. With modest increases in temperature, the A-U^f signal becomes infinitely broad. This occurs between 310 and 320 K for the A-U^f 12-mer and at approximately 313 K for Pribnow U^f. In both oligomers, the broadening of the A-U^f signals occurs at temperatures lower than that required for adjacent A-T base pairs, or for the analogous A-T pair in the control oligomers. Line broadening for resonances of the other base pairs occurs at temperatures

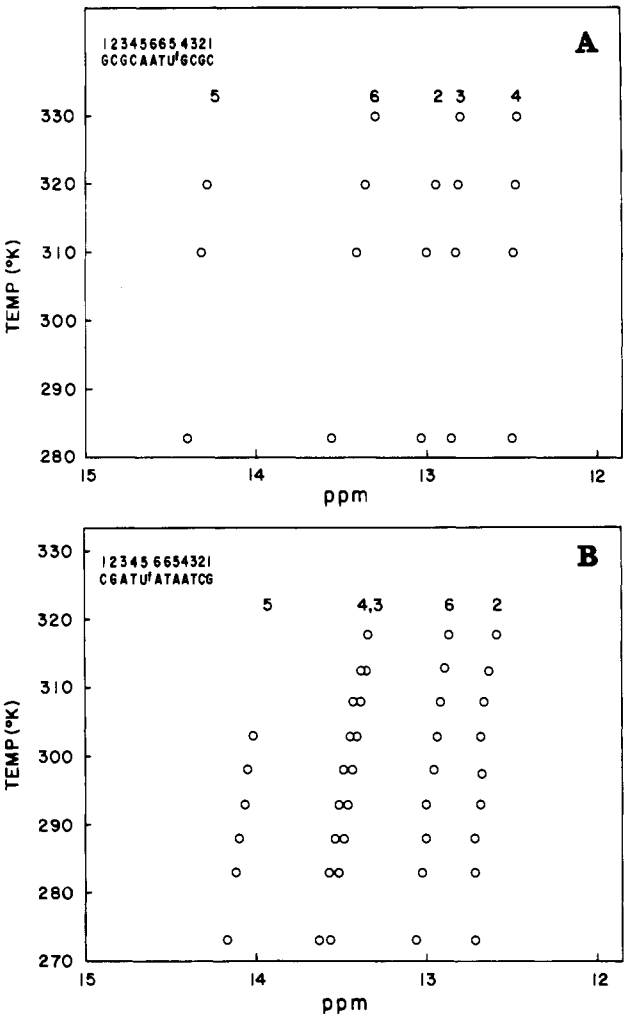

FIGURE 5: Premelting transitions of A-U^f and Prib U^f. The change in chemical shift of the imino proton resonances with increasing temperature is shown. All samples were in 1 mM cacodylate, 100 mM NaCl, and 1 mM EDTA, pH 7.3. (A) A-U^f; (B) Prib U^f.

similar to those noted in the control oligomers.

Base Pair Opening Rates. Base pair opening rates can be derived directly from temperature-induced line broadening only if the exchange process is open-limited. When aliquots of a solution containing 300 mM Tris, 1 mM cacodylate, 100 mM NaCl, and 2 mM EDTA, pH 7.3, were added to any of the oligomers dissolved in 1 mM cacodylate, 100 mM NaCl, and 2 mM EDTA, pH 7.3, broadening of the imino resonances was

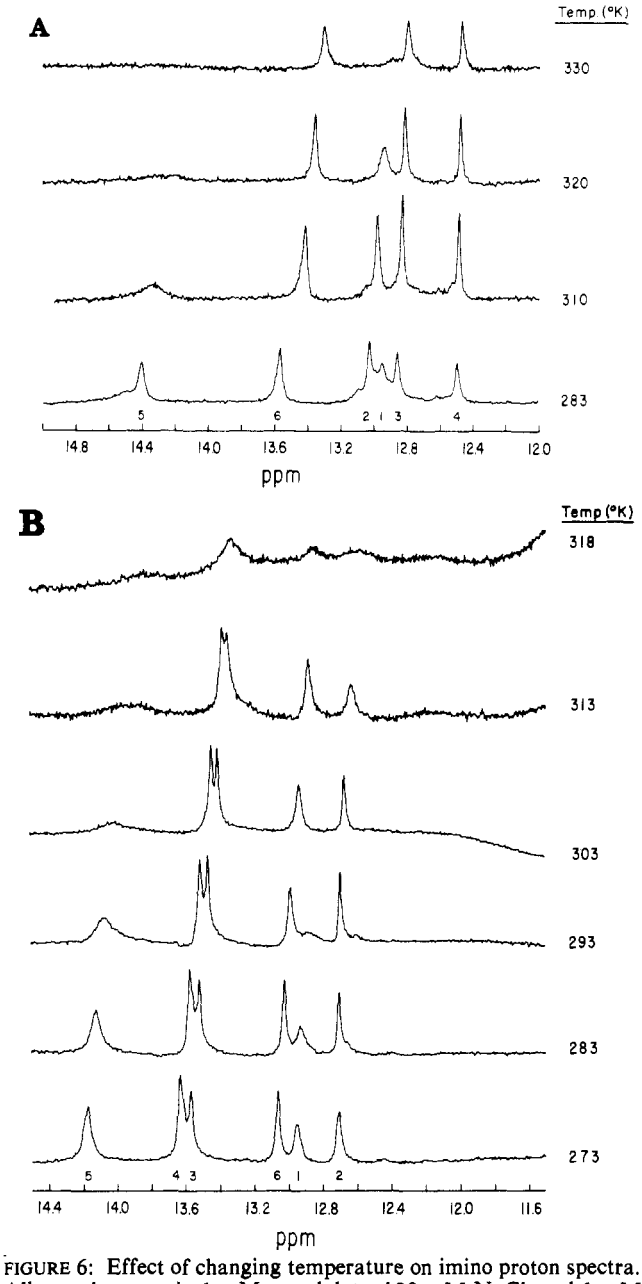

FIGURE 6: Effect of changing temperature on imino proton spectra. All samples were in 1 mM cacodylate, 100 mM NaCl, and 1 mM EDTA, pH 7.3. (A) A-U^f; (B) Prib U^f.

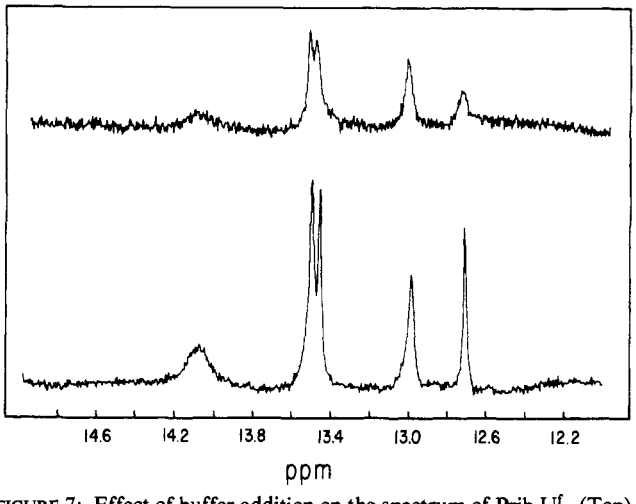

FIGURE 7: Effect of buffer addition on the spectrum of Prib U^f. (Top) Same buffer as for temperature study. (Bottom) After addition of 50 mM Tris.

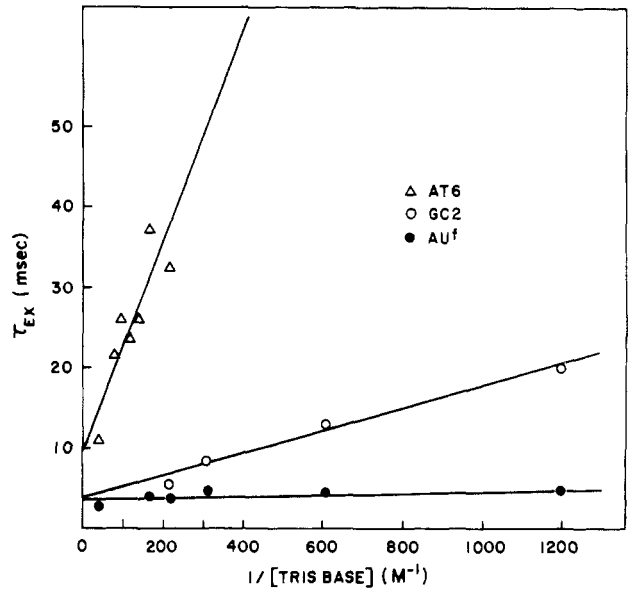

FIGURE 8: Buffer catalysis of imino proton exchange in A-U^f 12-mer. Aliquots of Tris were added to the sample, 80 ODU/mL, in the control buffer. Probe temperature was 310 K.

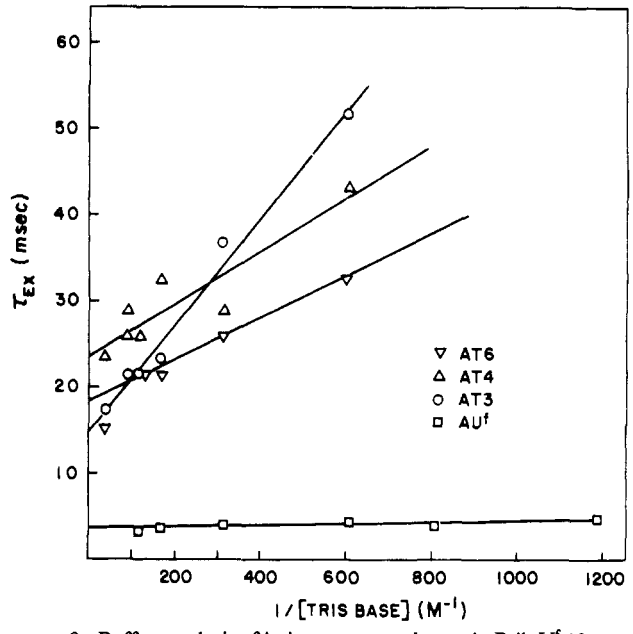

FIGURE 9: Buffer catalysis of imino proton exchange in Prib U^f 12-mer. Aliquots of Tris were added to the sample, 250 ODU/mL, in the control buffer. Probe temperature was 298 K.

observed (Figure 7). This result was found for all of the imino proton signals. Consequently, buffer catalysis occurs in this system, and the exchange reaction is not open-limited.

It has been shown that base pair opening rates for DNA duplexes can be derived by determining t_{ex} for the base pair at several buffer concentrations and extrapolating to infinite buffer (Leroy et al., 1985a,b). This yields t_0, the lifetime of the closed base pair. The results of this study, for the A-U^f 12-mer and Pribnow U^f 12-mer, are shown in Figures 8 and 9. As shown in these figures, the A-U^f base pair is relatively insensitive to buffer concentration in both oligomers and has a closed lifetime of ~3.5 ms in the A-U^f 12-mer at 310 K and ~3.5 ms in the Pribnow U^f 12-mer at 298 K. The other three A-T base pairs in the Pribnow U^f 12-mer have lifetimes ranging between 15 and 24 ms at 298 K, while the A-T base pair of the A-U^f 12-mer is 10 ms at 310 K. The shorter lifetime of the A-U^f base pair is also observed when it is

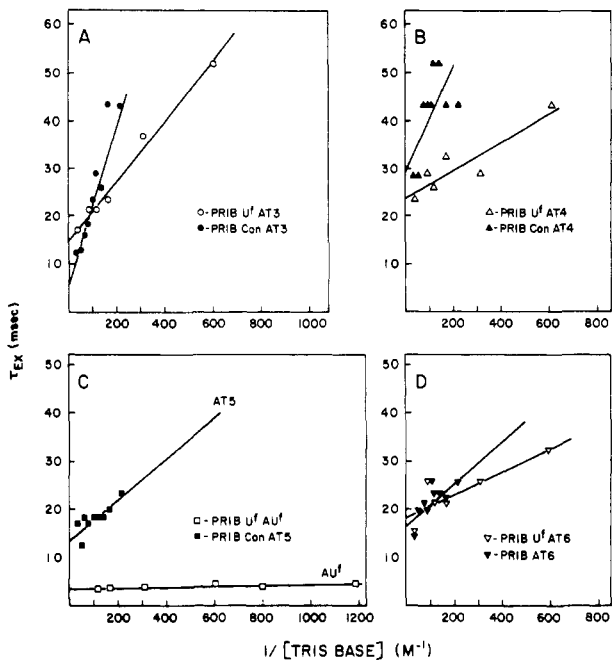

FIGURE 10: Comparison of base pair lifetimes of the Pribnow U^f and control duplexes. Aliquots of Tris were added to the sample, 250 ODU/mL, in the control buffer. Probe temperature was 298 K.

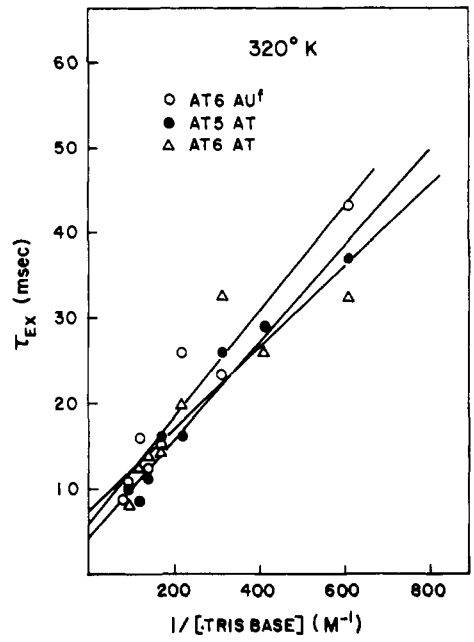

FIGURE 11: Comparison of the A-T base pairs of the A-U^f 12-mer and control. Aliquots of Tris were added to the sample, 80 ODU/mL, in the control buffer. Probe temperature was 320 K.

compared to the A-T base pair lifetimes of the control duplexes. Figure 10 shows the concordance among the base pair lifetimes of the base pairs of the Pribnow control of U^f oligomers. Only the A-U^f base pair opens more rapidly than the analogous position of the control oligomer. The A-T base pairs of the control and A-U^f 12-mers are also equivalent, as shown at 320 K in Figure 11. No attempt was made to quantitatively distinguish the individual A-T base pair lifetimes because of the differences that were observed were small, in relation to the scatter of the data.

Discussion

It has been suggested that the substitution of an electronegative halogen atom on the pyrimidine ring, by increasing the acidity of the N-3 position, may lead to mispairing with guanine (Freese, 1959). Studies of this process with 5-bromouracil have been carried out (Lasken & Goodman, 1984a,b); however, direct extrapolation to the U^f system is not possible due to the different electronic structure of the U^f anion (Wempen & Fox, 1964). The change in chemical shift with pH of a nucleus attached to a heterocyclic ring has been used to determine the pK_a of the ring, and good agreement with potentiometric methods has been found (Byrd et al., 1978; Kremer et al., 1980). The results of the titration study demonstrate that the pK_a of the U^f ring when incorporated into a trinucleotide does not differ much from that of the free nucleoside. These results are similar to those found with poly(U^f) (Loomis & Alderfer, 1985). Further, the nature of the neighboring bases does not affect the p*Ka*. Ionized U^f residues will thus exist in DNA at physiological pH, and this could lead to mispairing with guanine.

NMR spectroscopy has been utilized to compare the dynamics of DNA helices containing base pair mismatches (Patel et al., 1982b, 1984; Pardi et al., 1982), unpaired bases (Pardi et al., 1982; Patel et al., 1982c), and helices modified by methylation or interaction with drugs (Pardi et al., 1983; Fazakerley et al., 1985; Quignard et al., 1985; Hartog et al., 1985) with those of control helices. The ^{19}F data imply the presence of ionized U^f residues under the conditions of this study. The presence of this ionized base could affect the stability of the involved base pair, the stability of the other base pairs, or the overall dynamics of the helix.

The imino protons involved in the interbase hydrogen bonds can be observed to resonate between 12 and 15 ppm, a region uncomplicated by other resonances (Kearns et al., 1971). The currently accepted theory for the mechanism of exchange of these protons with solvent involves a two-step process (Teitelbaum & Englander, 1975).

$$\text{N–H}\cdots\text{N} \underset{}{\overset{K_{op}}{\rightleftharpoons}} \text{N–H N} \qquad (1)$$

$$\text{N–H} + \text{B–H}^* \overset{K_{ex}}{\rightleftharpoons} \text{N–H}^* + \text{B–H} \qquad (2)$$

In this mechanism, K_{op} represents the opening rate of the individual base pair ("breathing"), and K_{ex} is the rate of exchange of the proton from the opened base with solvent. The theory requires that no exchange occurs from the closed state and that there are no partially open configurations.

In the case where $K_{ex} \gg K_{op}$ ("open-limited"), K_{op} is the rate-determining step. Under such conditions, measurement of the line width at half-height, or saturation recovery studies, as a function of temperature accurately yields the opening rate of the base pair (Pardi et al., 1982). Recent work, however, has demonstrated that buffer catalysis of the exchange of imino protons occurs so that the open-limited conditions does not obtain (Leroy et al., 1985a,b). Under such circumstances, the change in line width with temperature can yield only a qualitative estimate of the base pair stability, and the derived opening rates represent only a minimum rate (Leroy et al., 1985a,b). In addition, all such previous work has been done with bases having a similar pK_a for the ring nitrogen (~9.3). From the equations (Leroy et al., 1985a,b) used to demonstrate the presence of buffer catalysis, it would be expected that lowering the pK_a of the ring would change the response to the catalyst.

The temperature-dependent line broadening does provide a useful qualitative comparison for the oligomers in this study. With the A-U^f resonance excluded the imino proton resonances of the A-U^f 12-mer and the Pribnow U^f 12-mer broaden at temperatures similar to those found for their respective controls. This implies that the presence of a single A-U^f base pair,

in a full turn of double helix, does not destabilize the entire helix. The upfield shift of the imino proton resonances with increasing temperature has been ascribed, in the control duplexes, to a premelting transition involving the unwinding of the helix with decreased stacking. The U^f-containing oligomers underwent the same temperature-dependent change as the control oligomers. This implies that the oligomers undergo similar premelting transitions.

The A-U^f resonances in both helices are far broader than the A-T resonances. This implies, on a qualitative basis, that the A-U^f imino proton exchanges more rapidly than the A-T. This is consistent with the ^{19}F data, which demonstrate that the U^f ring, when incorporated into an oligonucleotide, is significantly more acidic than that of thymine.

The control oligomers, and the U^f-containing ones, showed broadening of the imino proton resonances when Tris was added to the solution. Therefore, none of these protons can be considered to exist in an open-limited state. Consequently, the actual lifetimes of the closed state can be derived only from a buffer catalysis study (Leroy et al., 1985a,b). Although the buffer catalysis method holds out the promise of quantitative determinations of base pair lifetimes, some caution must be employed in interpreting the results. At buffer concentrations that induce minimal catalytic exchange, the observed changes in line width are small and difficult to measure accurately. As the method of extrapolating to infinite buffer concentration employs an inverse relationship, such points may be disproportionately weighted. Further, the line width in the absence of exchange must be estimated, introducing another potential source of error. Bearing these caveats in mind, useful information can be obtained. Extrapolation to infinite buffer concentration yields shorter lifetimes than have been reported when an open-limited condition was assumed. This is consistent with findings reported for other helices (Leroy et al., 1985a,b). There was excellent concordance among the response to buffer of the A-T base pairs of the control and U^f-containing duplexes. Only at base pair 3 was the lifetime of the base pair significantly shorter in one of the duplexes. It is notable that in this case the more rapidly opening base pair was found in the control helix, which also argues against any relative destabilization of the Pribnow U^f oligomer. Consequently, the presence of this abnormal base pair does not significantly destabilize nearby base pairs. This was true in the A-U^f 12-mer even at 320 K, a temperature at which the U^f imino proton exhibits fast exchange with solvent. This result differs from the observations that have been made in systems containing methylated cytosine, or adenosine, in which neighboring base pair effects were present (Mirau & Kearns, 1984; Quignard et al., 1985). Due to the differing temperatures of the experiments, it is not possible to make direct comparisons of the A-T lifetimes in the A-T 12-mer and the Pribnow 12-mer. In both oligomers, the A-U^f base pair opens faster than any of the A-T base pairs. The lifetime of the A-U^f imino proton in the A-U^f 12-mer is actually closer to that of the G-C 2 imino proton, an exterior base pair affected by fraying, than to the other interior base pairs. Therefore, the ionization of the U^f ring destabilizes the A-U^f base pair but, at pH 7.3, does not affect the stability of the other base pairs.

The occurrence of buffer catalysis is predicted by the equation:

$$k_{tr} = k_D(1 + 10^{-\Delta pK})$$

where k_{tr} is the transfer rate, k_D is the collision rate, and pK is the difference between the pK_a's of the buffer and the imino proton, respectively. On the basis of this equation, decreasing the pK_a of the proton undergoing catalysis should increase the susceptibility to catalysis. Catalysis should be apparent at low buffer concentration, and there should be minimal change in t_{ex} with increasing buffer concentration. The A-U^f resonance is already quite broad in the absence of buffer and shows a response to buffer that is quite distinct from any of the A-T base pairs. Therefore, the theory is consistent with the observations made for the U^f imino proton.

This study has two principal implications for the mechanism of 5-fluorouracil-induced cytotoxicity. First, a significant fraction of U^f residues in DNA will be ionized at physiological pH, creating the possibility, at a chemical level, of mispairing. Second, the presence of U^f residues in DNA does not cause significant regional destablization of the helix. However, A-T-rich regions of DNA, such as the Pribnow box studied here, are known to be important as recognition sites for DNA binding enzymes. The presence of a more easily opened base pair may alter the recognition of some DNA regions. Finally, the lack of destabilization of the helix, as a whole, and the similarity of the premelting transitions in the control and U^f helices suggest that 5-fluorouracil may be useful as a minimal perturbation NMR probe in other DNA systems.

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Cytochrome *c* Orientation in Electron-Transfer Complexes with Photosynthetic Reaction Centers of *Rhodobacter sphaeroides* and When Bound to the Surface of Negatively Charged Membranes: Characterization by Optical Linear Dichroism†

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ABSTRACT: Heme orientation with respect to the membrane normal has been measured for the cytochromes *c* and c_2 bound to photosynthetic reaction centers from *Rhodobacter* (*Rhodopseudomonas*) *sphaeroides* R-26 in reconstituted phosphatidylcholine vesicles. Previous kinetic studies have suggested that each cytochrome may bind in two configurations, which lead to either rapid or slow electron transfer to the flash-oxidized reaction center bacteriochlorophyll dimer. The rapid oxidation of cytochrome *c* is ~20-fold slower than that of cytochrome c_2. Optical linear dichroism measurements reported here show that, for both cytochromes, only the population undergoing rapid oxidation is dichroic. A stoichiometry of 0.5 dichroic cytochrome *c* or c_2 is found bound per reaction center. Prominent differences between the dichroism of the cytochrome c_2–reaction center complex and that of the cytochrome *c*–reaction center complex show that heme orientation differs in the two cases. The dichroism of cytochrome *c* bound to the reaction center can be distinguished from its dichroism when bound to the surface of negatively charged membranes. Analysis of the dichroism spectra suggests that, for cytochrome c_2, the heme is tilted 7°–8° closer to the membrane normal and rotated by 32° compared to the cytochrome *c*–reaction center complex. The dichroism spectra are consistent with the notion that the site on the cytochrome *c* surface that binds to the reaction center is the same site that binds to mammalian cytochrome *c* oxidase and reductase. However, a different locus is implicated on the surface of cytochrome c_2. These data suggest that although the tertiary structures of the cytochromes are homologous, the binding site is not conserved. These differences in cytochrome orientations may be in part responsible for the differences in the rate of electron transfer to the reaction center.

The mitochondrial cytochromes *c* and bacterial cytochromes c_2 form a single structural class (Meyer & Kamen, 1982). Cytochromes from both families show a similar conserved protein folding pattern and solvent-exposed heme edge. Yet variations in the specific amino acid sequences produce cytochromes that vary in net charge and redox potential (Bartsch, 1978; Meyer & Kamen, 1982; Meyer et al., 1983). It is expected that these variations optimize electron transfer by

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