Kinetics for Exchange of the Imino Protons of the d(C-G-C-G-A-A-T-T-C-G-C-G) Double Helix in Complexes with the Antibiotics Netropsin and/or Actinomycin†

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ABSTRACT: The lifetimes for exchange of the imino protons in the dodecanucleotide d(C-G-C-G-A-A-T-T-C-G-C-G) upon binding of netropsin and/or actinomycin have been measured by proton nuclear magnetic resonance experiments. At high temperature these lifetimes were found to measure the lifetimes for opening of the base pairs in the double helix. Comparison of the opening rates in the dodecamer with those in the complex with netropsin (which binds at the -A-A-T-T- sequence) shows that there is not only a large kinetic stabilization of the A·T base pairs at the binding site but also a significant stabilization of the G·C base pairs adjacent to the netropsin binding site. For the complex with actinomycin, which intercalates at the G-C sites in the double strand, the lifetimes of the base pairs at the binding site increase upon binding of actinomycin, and the A·T base pairs in the central core are slightly kinetically destabilized by the actinomycin binding. The activation energies for exchange of the imino protons were also measured in the complexes and indicate that the mechanism for exchange of the imino protons is individual base-pair opening, where one base pair opens independently of the others. The effects of drug binding on the dynamics of individual base pairs in a double-stranded helix are discussed.

Nuclear magnetic resonance (NMR)[1] experiments have been used to probe the conformation of drug–oligonucleotide complexes in the past mainly by observation of the chemical shift changes of the drug and/or nucleotide upon complex formation (Krugh & Nuss, 1979; Patel, 1979, 1980). The kinetic behavior of drug–nucleic acid interactions has been studied on oligonucleotides or polynucleotides by temperature-jump experiments (Bresloff & Crothers, 1975; Davanloo & Crothers, 1976) and hydrogen–deuterium exchange method (Priesler et al., 1981). In this work we present results on the kinetics of opening of individual base pairs in drug–oligonucleotide complexes as studied by proton NMR.

Actinomycin D is an antibiotic drug which has been shown to intercalate in double-stranded DNA specifically at G(3′–5′)C sites (Meienhofer & Atherton, 1977; Sobell, 1973; Krugh et al., 1977). Netropsin is a peptide antibiotic which binds in the minor groove of DNA duplexes specifically at A·T-rich regions (Zimmer, 1975; Wartell et al., 1974). The structure of the dodecanucleotide d(C-G-C-G-A-A-T-T-C-G-C-G), referred to as the 12-mer, has been solved by X-ray crystallographic techniques (Wing et al., 1980; Drew & Dickerson, 1981). The conformation of this molecule in solution has been studied by NMR experiments (Patel et al., 1982a). The conformation of the 12-mer in complexes with netropsin and actinomycin bound separately, and simultaneously, has recently been studied by observation of the ^1H and ^{31}P NMR chemical shifts in these complexes (Patel et al., 1981).

In this work we probe the dynamics of the 12-mer–antibiotic complexes by studying the kinetics of exchange of the imino protons of the double strand in the complexes. 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). We have recently studied the kinetics for exchange of the imino protons in the 12-mer and two related double helices, one with a G·T base pair and one with an extra adenine base (Pardi et al., 1982). The NMR of these three helices and also the NMR of the 12-mer–antibiotic complexes, including preliminary reports of some of the kinetic experiments performed here, have recently been reviewed (Patel et al., 1982b). The kinetics for exchange in the free 12-mer are compared here with the 12-mer–antibiotic complexes to study the effect of drug binding on the dynamics of base-pair opening. The studies confirm results obtained from chemical shifts indicating perturbations at the binding sites and give additional information on changes in the dynamics of base pairs adjacent to the binding site. Comparing the lifetimes of exchange in the 12-mer and 12-mer–antibiotic complexes gives a more quantitative measure of the location and extent of perturbations induced by drug binding. The activation energies for exchange of the imino protons in the 12-mer–antibiotic complexes were determined and compared with those found in the 12-mer. The mechanism for exchange of the imino protons in the 12-mer–drug complexes is discussed.

Materials and Methods

The 12-mer was synthesized by a modified triester method followed by deprotection and purification (Patel et al., 1982a). The actinomycin and netropsin were obtained from Merck and Famitalia (Milan, Italy), respectively. The NMR samples contained 12.5 mg/mL d(C-G-C-G-A-A-T-T-C-G-C-G). Samples were made up in 0.2 mL of buffer containing 0.1 M phosphate and 2.5 mM EDTA with chemical shifts referenced to the internal standard 3-(trimethylsilyl)-1-propanesulfonate (DSS). Samples containing antibiotics had 2 equiv of actinomycin or 1 equiv of netropsin or both. The NMR experiments were performed on the HXS-360-MHz instrument at

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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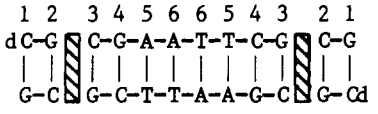
Table I: Lifetimes (ms) of Imino Protons in 12-mer-Actinomycin Complex



temp (°C)	proton 3	4	5	6
15			(190	185)
25			160	
30			185	
35			150	
40	170	135	100	
45	105	140	70	
50		135	50	
55	85	75	30	
60	70	40	17	

the Stanford Magnetic Resonance Laboratory. Temperature was controlled to ±1 °C and was calibrated by placing a thermocouple in an NMR tube (containing only D_2O) while it was in the probe. Data were collected on a Nicolet 1180 computer with 8K data points and a sweep width of ±5000 Hz. The large water signal was attenuated by the Redfield 214 pulse (Redfield et al., 1975) and the use of a tunable notch filter (Marshall et al., 1979). Saturation recovery experiments were performed as previously described (Pardi & Tinoco, 1982). Inversion recovery experiments were performed by using methods similar to the long pulse inversion recovery technique described by Early et al. (1980). Lifetimes of the saturation recovery experiments were analyzed as previously described (Pardi & Tinoco, 1982), and the inversion recovery analysis was done by using a three-parameter nonlinear least-squares fit to the inversion recovery function. Errors in the lifetimes are not more than ±20%. No significant differences were found in the lifetimes measured by the two techniques. Typically 10–15 different delay times were taken with 200–250 scans for each point.

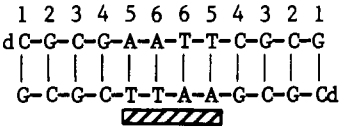
Results

Figure 1 shows the 360-MHz spectra of the low-field resonances of the 12-mer, the 12-mer–actinomycin complex, the 12-mer–netropsin complex, and the 12-mer–netropsin–actinomycin complex. The assignment of the imino protons in these complexes has been previously discussed (Patel et al., 1981, 1982b). In this paper we will be discussing only imino protons from base pairs 3–6 as the protons from base pairs 1 and 2 exchange too rapidly to allow sufficient data to be obtained in the presence or absence of bound antibiotics. The chemical shifts vs. temperature for the imino protons in G·C base pairs in all the complexes is shown in Figure 2. A semilog plot of data used to determine the relaxation lifetimes is shown in Figure 3.

For the 12-mer–actinomycin complex the resonances for the A·T base pairs 5 and 6 overlap at all temperatures, except 15 °C where two peaks are observed which have equal lifetimes for exchange. The temperature dependence of the lifetimes for the imino protons in the 12-mer–actinomycin complex are given for base pairs 3–6 from 15 to 60 °C in Table I. Arrhenius plots for the imino protons in this complex are given in Figure 4. The activation energies for exchange of imino protons 5 and 6 are given in Table IV.

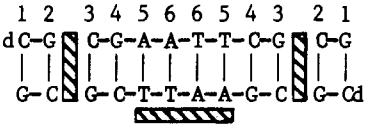
The lifetimes of the imino protons in the 12-mer–netropsin complex from 15 to 80 °C are given in Table II. Arrhenius plots for these protons are given in Figure 5. The activation energies determined from these plots are given in Table IV.

Table II: Lifetimes (ms) of Imino Protons in 12-mer-Netropsin Complex



temp (°C)	proton 2	3	4	5	6
15				320	325
20				340	375
25				305	370
30				420	335
35	45	240	400	405	360
40				415	310
45	13	95		515	290
50			315	280	230
55		50	225	225	165
60		35	210	235	130
65		17	150	135	105
70			100	120	85
75			60	75	65
80			35	60	40

Table III: Lifetimes (ms) of Imino Protons in 12-mer-Netropsin-Actinomycin Complex



temp (°C)	proton 3	4	5	6
20				160
25				180
35				180
45		155	175	160
55		130	130	135
60		85	75	75
65	55	65	55	55
70		35	50	45
75		35	25	25
80	12	18	19	19

Table IV: Activation Energies (kcal/mol) for Exchange of Imino Protons in 12-mer–Antibiotic Complexes

complex	proton 3	4	5	6
12-mer alone[a] (pH 6)			14 ± 2	15 ± 2
12-mer-actinomycin			17 ± 3	17 ± 3[b]
12-mer-netropsin	18 ± 3	16 ± 2	12 ± 2	12 ± 2
12-mer-netropsin-actinomycin		17 ± 3	18 ± 3	17 ± 3

[a] Data taken from Pardi et al. (1982). [b] Resonances from base pairs 5 and 6 overlap in this complex (see text).

For the 12-mer–netropsin–actinomycin complex the lifetimes of the imino protons from 20 to 80 °C are given in Table III. Arrhenius plots for the imino protons in base pairs 4–6 are given in Figure 6; the corresponding activation energies for exchange of imino protons are given in Table IV.

Discussion

We will use the chemical shifts of the imino protons of the 12-mer upon complex formation with actinomycin and/or netropsin to obtain information on the extent of binding of these drugs to the 12-mer duplex. The temperature depen-

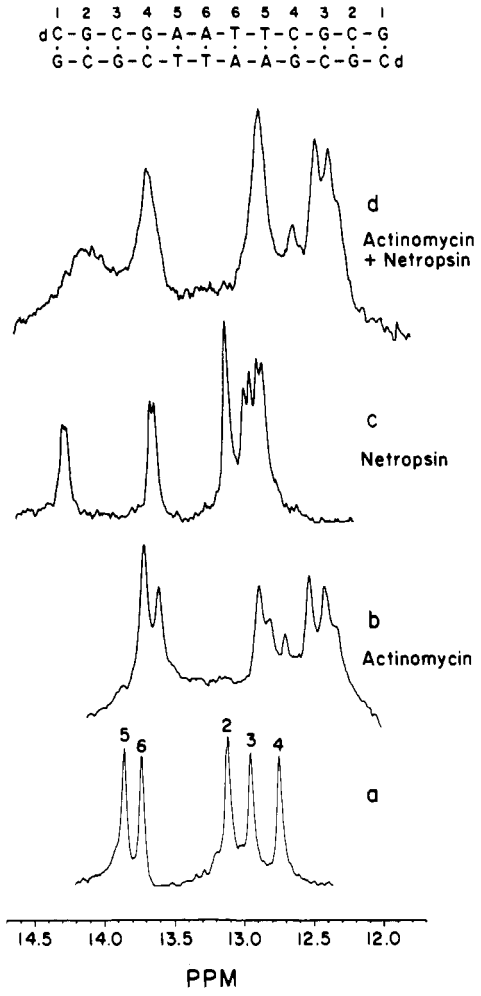


FIGURE 1: 360-MHz NMR spectra, low-field region, of (a) the 12-mer duplex, 20 °C, pH 6.8, (b) the complex with the 12-mer duplex and 2 equiv of actinomycin, 15 °C, pH 7, (c) the complex with the 12-mer duplex and 1 equiv of netropsin, 20 °C, pH 7, and (d) the complex with the 12-mer duplex with both 2 equiv of actinomycin and 1 equiv of netropsin, 20 °C, pH 7.

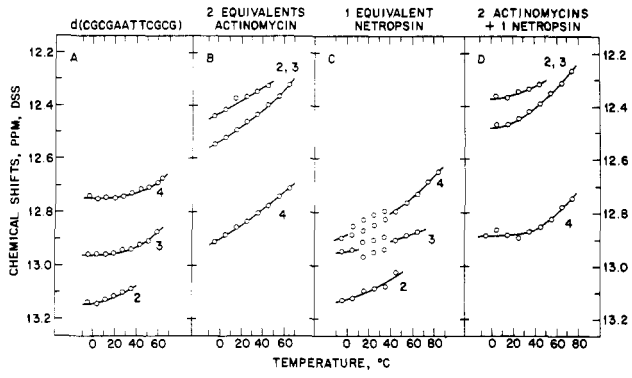


FIGURE 2: Temperature dependence of the G·C imino protons on base pairs 2–4 in (A) the 12-mer, (B) the 12-mer–actinomycin complex, (C) the 12-mer–netropsin complex, and (D) the 12-mer–netropsin–actinomycin complex.

dence of the chemical shifts of the G·C imino protons in the 12-mer alone and the 12-mer in a complex with actinomycin is shown in parts A and B of Figure 2, respectively. As seen in Figure 2B the imino protons on base pairs 2 and 3 shift upfield by at least 0.4 ppm upon complex formation with two actinomycins per 12-mer. If the free 12-mer and the 12-mer in the complex were in fast exchange on the NMR time scale, then we would expect to observe a weight-average chemical shift for the imino protons. Because of the large shift upon

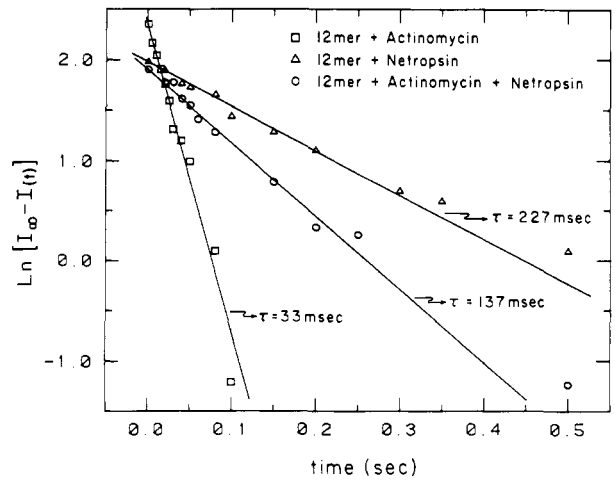


FIGURE 3: Semilog plot of data used to determine the relaxation lifetimes for the three antibiotic complexes at 55 °C. The value of ln $[I_\infty - I_{(t)}]$ and the parameters used to draw the lines were determined from an exponential fit of the measured intensities as described in Pardi & Tinoco (1982).

binding we conclude that if the free and bound 12-mer are in fast exchange, then, even at high temperature (60 °C), the concentration of the free 12-mer is very small. If there is slow exchange on the NMR time scale between free and bound 12-mer, then one would observe two separate peaks for the imino protons in these two states. We see no such behavior in the 12-mer–actinomycin complex and can thus still conclude that the concentration of free 12-mer must be small (<10%). Intermediate exchange is ruled out by the absence of broadening of the resonances except at the highest temperature where the imino protons do broaden due to chemical exchange of the imino protons with water. Thus for the 12-mer–actinomycin complex we observe that over the whole temperature range used in this study the concentration of free 12-mer is small (<10%) and that the 12-mer is essentially totally bound in the complex at all temperatures.

For the netropsin complex one A·T imino proton resonance shifts downfield at least 0.5 ppm from its position in the free 12-mer at all temperatures observed [see Figure 2 in Patel et al. (1981)]. We can again use this large difference in chemical shifts upon complex formation to conclude that the concentration of free 12-mer is very small. A similar conclusion is reached for the 12-mer–netropsin–actinomycin complex as revealed by the chemical shift changes of the G·C imino protons in Figure 2 and the A·T imino proton in Figure 2 of Patel et al. (1981). Thus in all the 12-mer–drug complexes studied here the concentration of the free 12-mer is less than 10% of the total, and therefore the observed NMR parameters arise essentially from the 12-mer in the complex.

Spin–Lattice Relaxation vs. Chemical Exchange. In previous studies on the 12-mer we found that at high temperature, chemical exchange with water was the dominant relaxation mechanism for the imino protons in the duplex (Pardi et al., 1982). The magnetic spin–lattice relaxation, T_1, is dominant in the 12-mer only at temperatures lower than 30 °C. Similar results are observed in the 12-mer–antibiotic complexes studied here. For the 12-mer–netropsin complex the observed lifetimes increased sharply with decreasing temperature from 80 °C down to around 45 °C where they level off (see Table II). This is more clearly seen in the Arrhenius plots in Figure 5. We will concentrate on the high temperature range where exchange is the dominant relaxation mechanism and so will use temperatures from 45 to 80 °C for analysis of exchange in the 12-mer–netropsin complex. Table I and Figure 4 show that

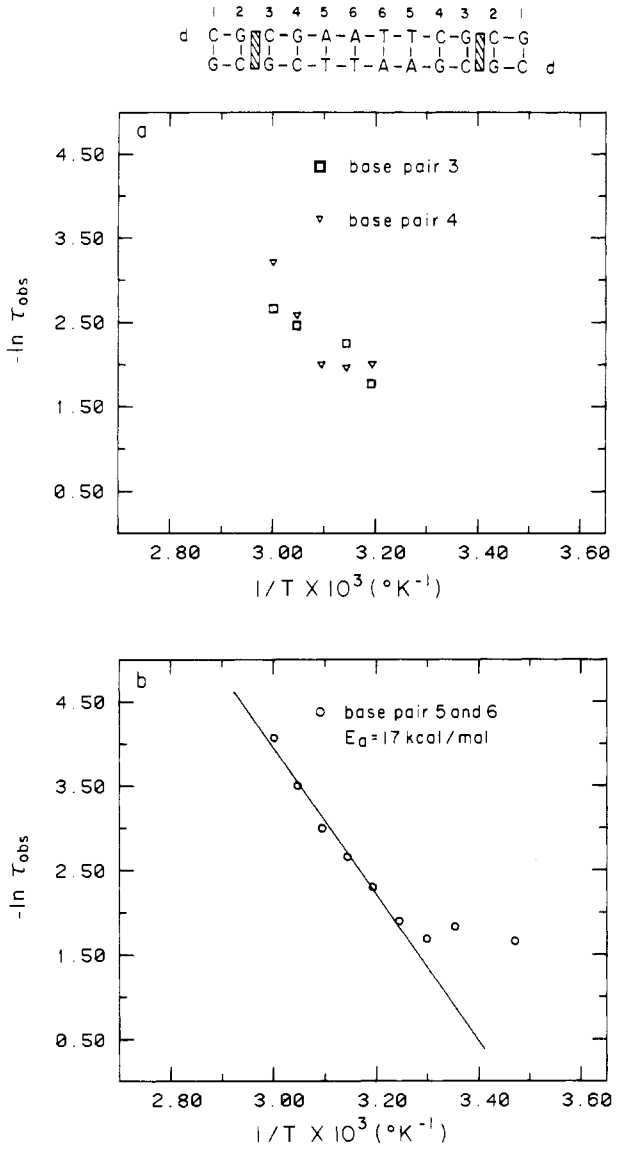


FIGURE 4: Arrhenius plots for the observed lifetimes of the 12-mer–actinomycin complex for (a) the G·C base pairs and (b) the A·T base pairs. The activation energy was calculated for temperatures of 35 °C and above.

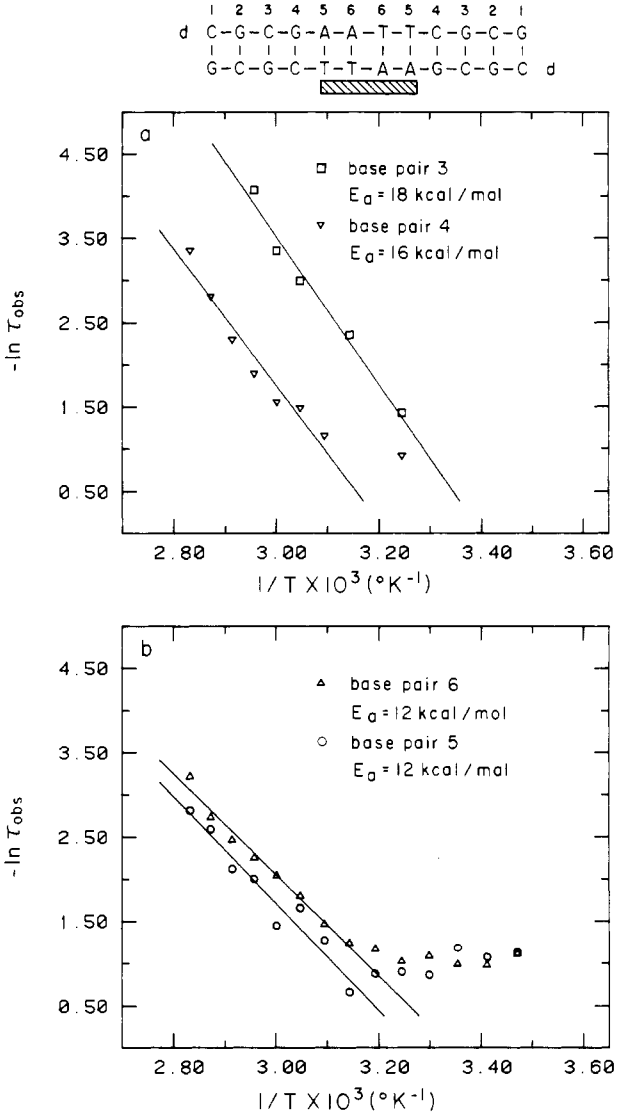


FIGURE 5: Arrhenius plots for the observed lifetimes of the 12-mer–netropsin complex for (a) the G·C base pairs and (b) the A·T base pairs. The activation energy was calculated for temperatures of 45 °C and above.

similar behavior is observed for the less stable 12-mer–actinomycin complex, and we will use temperatures from 35 to 60 °C for analysis of chemical exchange lifetimes of the imino protons. The behavior of the observed lifetimes in the 12-mer–netropsin–actinomycin complex, as seen in Table III and Figure 6, indicates that chemical exchange is the dominant relaxation pathway for the imino protons from 55 to 80 °C.

Although the lifetimes measured at high temperature depend predominantly on chemical exchange, they will have a contribution from magnetic spin–lattice relaxation. To estimate the maximum contribution from magnetic relaxation, we assume that the measured lifetimes at low temperature are completely determined by magnetic spin–lattice relaxation and that these lifetimes are independent of temperature. The magnetic spin–lattice lifetimes for a similar oligonucleotide were found to increase with increasing temperature (Early et al., 1981); therefore our assumptions will lead to an upper limit for a correction to the chemical exchange rates. Figures 3–5 show that good estimates for the magnetic spin–lattice relaxation times can be determined for A·T base pairs 5 and 6 in all the 12-mer–antibiotic complexes; however, it is more difficult to determine the lifetimes for G·C base pairs 3 and 4. Therefore, contributions of the magnetic spin–lattice relaxation times to the high-temperature data for the G·C base pairs were determined by using both the lowest temperature G·C data and also magnetic spin–lattice lifetimes determined for the A·T base pairs.

Upon making these corrections we find that the magnitudes of the chemical exchange lifetimes increase but that the relative magnitudes are not changed. Making these corrections to the lifetimes will also increase the values of the activation energies. However, the maximum effect of magnetic spin–lattice processes does not change any of the comparisons or conclusions made in the following sections. We discuss the measured lifetimes and activation energies determined from those lifetimes rather than corrected ones, because of the uncertainty in estimating the magnetic spin–lattice effects.

Exchange Lifetimes in the 12-mer–Antibiotic Complexes. We have recently shown that the imino protons of base pairs 3–6 in the 12-mer duplex were in the open-limited region (Pardi et al., 1982), which means that every time the base pair opens the imino protons exchange with water. We will assume that base pairs 3–6 in the 12-mer–antibiotic complexes are also in the open-limited region and thus the lifetimes for exchange measure the kinetics of base-pair opening in these complexes.

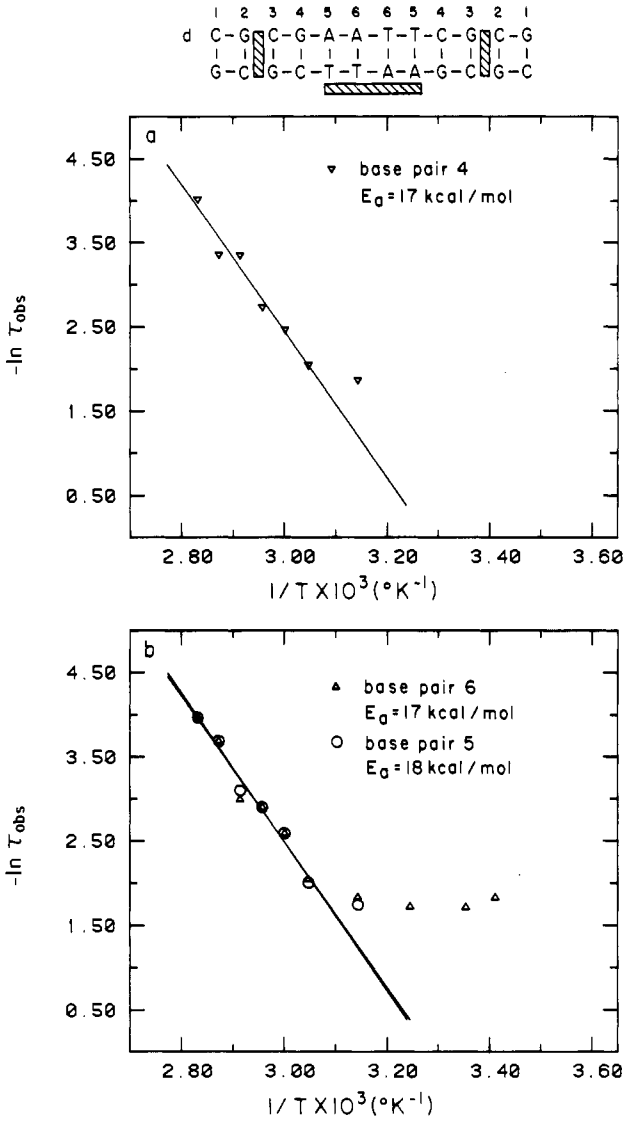


FIGURE 6: Arrhenius plots for the observed lifetimes of the 12-mer–netropsin–actinomycin complex for (a) the G·C base pair and (b) the A·T base pairs. The activation energy was calculated for temperatures of 55 °C and above.

Patel et al. (1981) have shown that two actinomycins bind per duplex in the 12-mer–actinomycin complex. Table I gives the lifetimes for exchange of the imino protons in this complex; they can be compared with the lifetimes for the free 12-mer (Pardi et al., 1982). The lifetimes for the overlapping A·T base pairs 5 and 6 are 30 ms in the complex at 55 °C; this is shorter than the 35 and 55 ms found for A·T base pairs 5 and 6 in the free 12-mer. The lifetime for G·C base pair 4 is essentially unchanged by actinomycin binding; at 55 °C it is 75 ms in the complex and 80 ms in the free 12-mer. However, the lifetime of G·C base pair 3 is strongly increased; at 55 °C it is 85 ms in the complex, but only 35 ms in the free 12-mer. Thus, binding actinomycin between G·C base pairs 2 and 3 decreases the rate of opening of G·C base pair 3 by over a factor of 2. It leaves G·C base pair 4 unchanged, and it slightly increases the rate of opening of A·T base pairs 5 and 6. Actinomycin kinetically stabilizes G·C base pair 3 at the binding site; it has little effect on base pair 4, but it destabilizes the two A·T base pairs 5 and 6. The effect of binding actinomycin at each G(3′–5′)C site is propagated into the helix, perhaps inducing some conformational changes in the A·T base pairs.

Netropsin is known to bind with a specificity for A·T regions in the minor groove of DNA duplexes (Zimmer, 1975; Wartell et al., 1974). It has been shown that in the complex only one netropsin is bound per 12-mer duplex (Patel et al., 1981). Netropsin, which lacks a center of symmetry, removes the 2-fold symmetry of the 12-mer as revealed by up to a 0.02 ppm chemical shift difference for base pairs 3–6 on different ends of the helix (Patel et al., 1981). However, no measurable difference in the exchange lifetimes for individual base pairs on different ends of the 12-mer helix in the netropsin complex was observed.

The lifetimes for the 12-mer–netropsin complex are given in Table II. First consider the A·T base pairs; the netropsin increases the lifetime for exchange of base pair 5 by over a factor of 6 from 35 ms in the free 12-mer to 225 ms in the 12-mer–netropsin complex at 55 °C. Base pair 6 is kinetically stabilized by a factor of 3 at 55 °C: the lifetime for exchange of the free 12-mer is 55 ms while that of the 12-mer–netropsin complex is 165 ms. Thus base pair 5 has the largest change in kinetics of the A·T base pairs upon netropsin binding. Base pair 5 has also been found to have a very large downfield shift of the imino proton resonance upon netropsin binding (Patel et al., 1981). What is the effect on base pairs adjacent to the binding site? The lifetime for exchange of G·C base pair 3 in the free 12-mer at 55 °C is 35 ms; comparing this with a lifetime of 50 ms in the 12-mer–netropsin complex shows an increase of about 50% upon drug binding. However, the lifetime for exchange of G·C base pair 4 is increased by almost a factor of 3 upon netropsin binding: from 80 ms for the free 12-mer to 225 ms in the 12-mer–netropsin complex. Thus we see that binding of netropsin at the central core of the 12-mer duplex affects the dynamics of the G·C base pairs which are distant from the binding site. The binding of netropsin is known to span two to four base pairs, therefore its binding presumably includes all four A·T base pairs. It is interesting to note that upon complex formation the relative increase in the lifetime for opening of the G·C base pair at position 4 is as large as that found at the netropsin binding site at A·T base pair 6. This result could be due to netropsin directly interacting with the G·C site or could be an effect propagated down the helix from netropsin binding at the A·T sites.

The 12-mer has been shown to form a complex having two actinomycins and one netropsin simultaneously bound at adjacent G·C and A·T blocks (Patel et al., 1981). The lifetime in the 12-mer–netropsin–actinomycin complex at 55 °C for G·C base pair 4 is 130 ms compared to 80 ms in the free 12-mer; for A·T base pair 5 the lifetime is 130 ms in the complex compared to 35 ms in the free 12-mer, and A·T base pair 6 has a lifetime of 135 ms in the complex and 55 ms in the free 12-mer. Thus we observe that the lifetimes for exchange of all the imino protons have increased in the complex relative to the free 12-mer.

In comparing the lifetimes of the 12-mer–actinomycin complex with those in the 12-mer–netropsin–actinomycin complex, we see that the complex with both drugs bound has lifetimes for all the imino protons which are significantly larger than the corresponding lifetimes when only actinomycin is bound in the complex (130, 130, and 135 ms compared to 75, 30, and 30 ms; Tables I and III). Thus netropsin kinetically stabilizes base pairs 4–6 of the double helix when it binds to the 12-mer–actinomycin complex. Just the opposite is true for the 12-mer–netropsin complex; the lifetimes of the imino protons on base pairs 4–6 are greater in the 12-mer–netropsin complex than in the 12-mer–netropsin–actinomycin complex (225, 225, and 165 ms compared to 130, 130, and 135 ms; Tables II and III). Thus the actinomycin kinetically desta-

bilizes these base pairs in the double helix when it binds to the 12-mer–netropsin complex. Notice that the increase or decrease in the lifetimes of the 12-mer–netropsin–actinomycin complex compared to the 12-mer with only actinomycin or netropsin bound extends beyond the binding site of the second antibiotic.

Table III shows that the lifetimes of base pairs 4–6 are approximately the same at a given temperature in the 12-mer–netropsin–actinomycin complex. This result could be explained by a cooperative mechanism where all the imino protons exchange together. As will be discussed in the following section, the activation energies for exchange of the imino protons rule out a cooperative mechanism for exchange. Base pairs 4–6 in the 12-mer–netropsin–actinomycin complex thus exchange independently of one another but have similar rates for exchange.

Activation Energies for the 12-mer–Antibiotic Complexes. Activation energies for the imino protons in the 12-mer were previously measured, and it was shown that exchange takes place by an individual base-pair-opening mechanism (Pardi et al., 1982). The activation energies for the imino protons on base pairs 3–6 for the 12-mer–netropsin complex range from 12 to 18 kcal/mol (Table IV) and are similar to those found in the 12-mer under similar conditions. The activation energies for the A·T base pairs remained essentially the same; a 2–3 kcal/mol decrease from the free 12-mer was observed. The low values for activation energies indicate that an individual base-pair-opening mechanism is the dominant exchange process in this complex (Pardi et al., 1982).

The activation energies for base pairs 5 and 6 for the 12-mer–actinomycin complex are given in Table IV. Activation energies for G·C base pairs 3 and 4 were not calculated as it is difficult to determine from the data where chemical exchange becomes dominant. An Arrhenius plot of the data is shown in Figure 4a. The activation energies for base pairs 4–6 for the 12-mer–netropsin–actinomycin complexes are given in Table IV. The activation energies for both of these complexes are again similar to those found in the free 12-mer and indicate that exchange in both complexes takes place by an individual base-pair-opening mechanism. Thus a process involving a cooperative exchange of several base pairs does not seem to be an important mechanism in the kinetics for exchange of the imino protons for any of the 12-mer–antibiotic complexes studied here.

Conclusions

The lifetimes for exchange of the imino protons have been investigated in complexes of a dodecanucleotide double strand with the antibiotics netropsin and/or actinomycin. These lifetimes of base-pairing protons in the double strand upon drug binding give information on the changes in the kinetic stability of base pairs at the binding site and at different distances from the binding site. The lifetimes were measured by saturation recovery experiments or inversion recovery experiments and were shown to correspond at high temperatures to lifetimes of chemical exchange of the protons. Comparison with previous work on the 12-mer shows that the exchange of each imino proton is a measure of the rate of opening of each base pair in the complex. That is, every time the base pair is broken, the imino proton exchanges with water.

Actinomycin intercalates at the G(3′–5′)C site between base pairs 2 and 3 in the 12-mer (Patel et al., 1981). G·C base pair 3 at the binding site was significantly stabilized by actinomycin binding, but the lifetime of G·C base pair 4 was unaffected. The binding kinetically destabilized the A·T base pairs at the central core of the 12-mer slightly. However, in the 12-mer–netropsin complex the lifetimes of all the base pairs increase upon drug binding. The netropsin is known to bind in the minor groove at A·T-rich sites in DNA and binds in the central A·T core of the 12-mer. There is a large increase in the lifetime for exchange of G·C base pair 4 next to the A·T netropsin binding site, indicating that the netropsin may interact with this G·C residue, or at least induces a perturbation in the helix which stabilizes this base pair. Comparing the lifetimes of the imino protons in the complex when both drugs are bound with those when only one drug is bound, one sees that the binding of netropsin stabilizes the A·T base pairs as well as G·C base pair 4 relative to the 12-mer–actinomycin complex, whereas the binding of actinomycin to a 12-mer–netropsin complex destabilizes all of these base pairs. It is clear that the effects of binding of a drug at a specific site in the helix can be propagated along the double strand and alter the dynamics for opening of base pairs beyond the nearest-neighboring residues.

The activation energies for exchange of the imino protons in the 12-mer complexes were studied by measuring the temperature dependence of the exchange rates. The activation energies are not dramatically different from those found in the free 12-mer, with values ranging from 12 to 18 kcal/mol. These relatively small values for exchange of imino protons in the three 12-mer–antibiotic complexes studied here show that exchange takes place by an individual base-pair-opening mechanism. The activation energies change only slightly with and without bound drugs, indicating that the open states may have quite similar structure.

Studies on the kinetics of binding of the antibiotics netropsin and/or actinomycin to the 12-mer would be extremely useful in probing the dynamics of these complexes in solution. Correlations between base-pair opening and drug dissociation in the complexes could then be studied to try to understand the general dynamics of drug–nucleic acid interactions.

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Isolation and Characterization of Two 5-Fluorouracil-Substituted *Escherichia coli* Initiator Methionine Transfer Ribonucleic Acids†

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ABSTRACT: *Escherichia coli* initiator methionine tRNA labeled in vivo with 5-fluorouracil (FUra) has been isolated and characterized. The tRNA, with essentially all its uracil and uracil-derived minor bases replaced by FUra, was purified by sequential chromatography, first on diethylaminoethylcellulose (DEAE-cellulose), at pH 8.9, followed by chromatography on Sepharose 4B, using a reverse salt gradient, then on DEAE-Sephadex A-50, and finally on benzoylated DEAE-cellulose. The last step resolved two FUra-substituted $tRNA_f^{Met}$-isoaccepting species, each with a specific activity over 1500 pmol/A_{260}. Kinetic analysis shows both are aminoacylated at the same rate; apparent K_ms for the two are 0.92 and 0.94 μM, compared with 1.7 μM for normal $tRNA_f^{Met}$. Chromatographic differences between the two forms of fluorinated $tRNA_f^{Met}$ persist after aminoacylation, and the two tRNAs are not interconverted by denaturation and renaturation. The isoacceptors have nearly identical nucleoside composition, and both contain 7-methylguanosine and 2′-*O*-methylcytidine as the only modified nucleosides. Analysis of complete RNase T_1 digests of the two methionine tRNAs shows that they differ in only one oligonucleotide. The sequence ^{20}FpApGp, derived from the dihydrouridine loop and stem region, which is found in one of the isoaccepting forms of the tRNA, is replaced by an oligonucleotide containing adenine and guanine, but no FUra in the other. A modified FUra, with the properties of a 5-fluoro-5,6-dihydrouracil derivative, is detected in this tRNA. ^{19}F NMR spectra of the two species of FUra-substituted initiator tRNA show 9–10 resolved resonances for the 12 FUra residues incorporated. The spectra differ primarily in the shift of one peak in the form lacking the sequence ^{20}FpApGp, from 4.8 ppm downfield from free FUra (=0 ppm) to 14.9 ppm upfield from the standard.

Transfer RNAs isolated from 5-fluorouracil (FUra)[1]-treated *Escherichia coli* are highly substituted with the fluoropyrimidine (Horowitz & Chargaff, 1959). Incorporated FUra replaces not only uridine but also uridine-derived minor nucleosides such as pseudouridine, ribothymidine, 5,6-dihydrouridine, and 4-thiouridine (Johnson et al., 1969; Lowrie & Bergquist, 1968; Kaiser, 1972). The resulting modification-deficient tRNAs, with a few exceptions (Ramberg et al., 1978), remain functional in protein synthesis and have proved to be quite useful for examining the role(s) of modified nucleosides in tRNA structure and function (Horowitz et al., 1974; Ofengand et al., 1974; Chinalli et al., 1978). Detailed studies with purified, highly substituted *E. coli* $tRNA_1^{Val}$ have shown that incorporation of 5-fluorouridine has little if any effect on the conformation of this tRNA (Horowitz et al., 1974). Furthermore, its rate of aminoacylation is normal (Horowitz et al., 1974), and it is active in all steps of protein synthesis in vitro (Ofengand et al., 1974).

In addition to examining the biochemical function of fluorine-substituted tRNAs, we have been exploring the use of fluorine-19 nuclear magnetic resonance as a probe of the molecular structure and dynamics of tRNA in solution. Fluorine NMR is well suited to such studies because of the high sensitivity of the ^{19}F nucleus and the large range of its chemical shifts. Since nuclear magnetic resonance permits observation of individual nuclei at different molecular sites, each FUra residue can, in principle, serve as a reporter of structural changes in its vicinity. Because FUra is distributed

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[1] Abbreviations: FUra, 5-fluorouracil; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; PEI–cellulose, poly(ethylenimine)–cellulose; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

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