Proton Exchange and Internal Motions in Two Chromomycin Dimer–DNA Oligomer Complexes†

Jean Louis Leroy,*,‡ Xiaolian Gao,§,∥ Maurice Guéron,‡ and Dinshaw J. Patel∥

Groupe de Biophysique, Ecole Polytechnique, 91128 Palaiseau, France, and Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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ABSTRACT: Previous structural studies on the complexes of the chromomycin (CHR) dimer with duplexes of d(A1-A2-G3-G4-C5-C6-T7-T8) and of d(A1-G2-G3-A4-T5-C6-C7-T8) in solution [one Mg(II) and two drugs per duplex] are extended to hydrogen exchange measurements. Exchange of the OH8 proton of chromomycin, measured by real time proton–deuterium exchange, is very slow and requires dissociation of the complex, whose lifetime is thus determined. The lifetimes and apparent dissociation constants of base pairs are deduced from the catalysis of imino proton exchange by ammonia. The four central base pairs, which interact with the CHR chromophores in the minor groove (Gao & Patel, 1990), may open within the complex, but the opening rate is less than in the free duplex by one to two orders of magnitude. The activation energy for base-pair opening and the differences between the lifetimes of adjacent pairs suggest that single base-pair opening is the predominant imino proton exchange pathway in all cases. In the symmetrical complex of chromomycin with the first duplex, the lifetimes of the central base pairs (G3·C6 and G4·C5) are in the same range (52 and 29 ms, respectively, at 38 °C). In the asymmetrical complex formed with the second duplex, the base-pair lifetimes in the G2-G3-A4-T5 segment that interacts with the chromophore moiety are strongly increased. That of G3·C6* is particularly long. Above 50 °C, exchange of the G3 imino proton is opening limited. The lifetimes of base pairs in contact with the E saccharide ring of the CHR are comparable to that of the free duplex. The kinetic data support and extend the structural results on the symmetrical complex and their extension to the asymmetrical one. Anomalous acid catalysis (up to pH 8!) of imino proton exchange of the guanine residue hydrogen bonded to the chromomycin chromophore points to still unknown features of these drug–DNA complexes.

The imino protons of individual base pairs provide well-resolved markers for the exploration of the internal motion of nucleic acids (Johnston & Redfield, 1977; Leroy et al., 1985). Their exchange with water involves a proton acceptor acting as a catalyst.

The base-pair lifetime may be obtained as the limit of the imino proton exchange time at infinite catalyst concentration, i.e., under conditions where exchange occurs at each opening event. The apparent dissociation constant of the pair is derived by comparing the exchange catalysis of the imino proton on the duplex with that of the free monomers. Systematic studies (Guéron et al., 1989) have established that ammonia, with its small size and high pK, is an efficient acceptor for imino protons.

Based on imino proton exchange catalysis, base-pair lifetimes and apparent dissociation constants have been reported for B-DNA (Kochoyan et al., 1987; Leroy et al. 1988a), B′-DNA (Leroy et al., 1988b), and Z-DNA (Kochoyan et al., 1990). By contrast, the effect of an exchange catalyst was not observed in earlier proton exchange measurements on drug–DNA oligomer complexes (Pardi et al., 1983), and therefore the imino proton exchange kinetics could not be interpreted in terms of internal motions.

We now apply ammonia catalysis to the study of proton exchange in two chromomycin–DNA oligomer complexes whose structures were determined previously and in which the drug binds as a dimer coordinated by Mg(II) in the minor groove of the deoxyduplex (Gao & Patel, 1989a,b, 1990).

The present work provides the first detailed measurements of proton exchange and base-pair kinetics in drug–DNA complexes. The examination of two complexes is too limited for generalization. Nevertheless, features common to both complexes are observed. Thus, binding of the Mg(II)-coordinated CHR[1] dimer does not prevent base-pair opening but slows it down by factors larger than 10, and the stability of base pairs interacting with the chromophore component of the drug is enhanced by one to two orders of magnitude with respect to the free duplexes. From exchange measurements, we also obtain the lifetime of each complex.

Experimental Procedures

NMR Samples. The oligonucleotides were synthesized on a 10-μmol scale by the β-cyanoethyl phosphoramidite method and purified by high-pressure chromatography as previously described (Gao & Jones, 1987; Kochoyan et al., 1990). The samples of the chromomycin–DNA oligomer complexes were prepared at the stochiometry of one Mg(II) and two drugs per duplex in 0.1 M NaCl, the reaction being monitored on the proton NMR spectrum. Ammonia was added to the samples from a 6.5 M stock solution, pH 8.8. The sample pH was checked with a microelectrode and adjusted as necessary with HCl or NaOH, 0.1–0.5 M. The pH and temperature of the

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* To whom correspondence should be addressed.

‡ Groupe de Biophysique, Ecole Polytechnique.

§ Present address: Glaxo Research Labs, 5 Moore Drive, RTP, NC 27709.

∥ Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University.

[1] Abbreviation: CHR, Chromomycin.

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samples are given in the text and in figure captions.

NMR Methods. The NMR measurements of the symmetrical Mg(II)-coordinated CHR–d($A_2G_2C_2T_2$) complex were performed mostly on Bruker AM400 and AM300 spectrometers, and those of the asymmetrical Mg(II)-coordinated CHR–d(AG_2ATC_2T) complex used the 360-MHz spectrometer built at Ecole Polytechnique. The H_2O signal was suppressed with the JR sequence (Plateau & Guéron, 1982; Guéron et al., 1991).

The methods for measuring imino proton exchange times and base-pair lifetimes have been described (Kochoyan et al., 1987; Leroy et al., 1988a). The exchange times longer than 1 min were determined in real-time exchange experiments by dilution of a concentrated protonated sample into D_2O. Exchange times shorter that 2 s were obtained from the rate of magnetization transfer from water (Forsen & Hoffmann, 1963). The exchange contribution of added ammonia was determined from the variation of the longitudinal relaxation, by using the relation $1/\tau_{ex} = 1/T_{1cat} - 1/T_{10}$, where T_{1cat} and T_{10} are the relaxation times measured with and without catalyst. The estimated precision is ±15% for the longer and ±50% for the shorter base-pair lifetimes and ±10% for the apparent dissociation constants.

Exchange Formalism. Exchange of the imino proton of a base pair requires disruption of the base pair. In the open state, the imino proton is transferred to water via a catalytic step involving a proton acceptor such as ammonia or the hydroxyl ion or a proton acceptor intrinsic to the nucleic acid.

Exchange Catalysis by an Added Catalyst (Kochoyan et al., 1987). The exchange contribution of the added catalyst is given by

$$\tau_{ex} = \tau_0(1 + k_{cl}/k_{tr}^{add}) \quad (1)$$

or

$$\tau_{ex} = \tau_0 + (1/K_d k_{tr}^{add}) \quad (2)$$

where τ_0 and K_d are the base-pair lifetime and dissociation constant, k_{cl} is the rate constant for closing of the pair, and k_{tr}^{add} is the rate of transfer to the catalyst in the open state. The latter is related to the added catalyst concentration [C] by (Eigen, 1964)

$$k_{tr}^{add} = k_{coll}[C]/(1 + 10^{\Delta pK}) \quad (3)$$

where k_{coll} is the collision rate, and ΔpK is the difference between the nucleoside and the catalyst pK.

If the accessibility to the imino proton in the open pair is perfect, k_{coll} is the same for the open pair and for the monomer except for the difference between the monomer and the duplex coefficients of translation diffusion (Guéron et al., 1989). Therefore,

$$k_{tr}^{add} \approx k_i/[1 + (M_c/M_n)^{1/3}] \equiv k_{i'} \quad (4)$$

where k_i is the exchange rate for the monomer at the same catalyst concentration, and M_c and M_n are the molecular masses of the catalyst and monomer.

In case of restricted access, we write $k_{tr}^{add} = \alpha k_i'$, where α is an accessibility factor that may depend on the exchange catalyst. Equation 1 may be written in terms of the apparent base-pair dissociation constant, αK_d:

$$\tau_{ex} = \tau_0 + 1/(k_i'\alpha K_d) \quad (5)$$

When the catalyst concentration is sufficiently high, i.e., $k_i'\alpha K_d \gg 1$, exchange occurs every time the base pair opens. At infinite catalyst concentration, τ_{ex} extrapolates to τ_0, the base-pair lifetime. At lower catalyst concentrations, the exchange rate is proportional to the fraction of the time during which the imino proton is exposed:

$$\tau_{ex} = 1/(k_i'\alpha K_d) \quad (6)$$

By comparison with the exchange time $1/k_i$ of the imino proton in the monomer, τ_{ex} yields the apparent base-pair dissociation constant αK_d. The lifetime of the open state, τ_{open}, is equal to $K_d\tau_0$, and the available parameter, $\alpha K_d\tau_0 \equiv \alpha\tau_{open}$, is denoted the "apparent lifetime" of the open-state.

The values of k_i were obtained from the imino proton line broadening, Δ, of the free monomers (thymidine and 2′–3′ guanosine) upon catalyst addition according to $k_i = \pi\Delta/[C]$ where [C] is the catalyst concentration. For OH^- at 0 °C, we find $k_i = 10^9$ and 2.5×10^9 $(s\cdot M)^{-1}$ for thymidine and guanosine, respectively. For NH3, we measure $k_i = 2 \times 10^8$ $(s\cdot M)^{-1}$ independently of temperature between 15 and 60 °C.

Exchange by Internal Catalysis (Guéron et al., 1987). In the absence of added catalyst, the imino proton exchange time τ_{AAC} remains finite. Exchange still takes place via transient base-pair opening, but it is catalyzed by a proton acceptor of the duplex itself, the cyclic nitrogen of the complementary base. Exchange occurs via proton transfer to a water molecule linking the imino proton to this group, as shown in Scheme I for a G·C pair. The equation corresponding to eq 2 is

$$\tau_{AAC} = \tau_0 + (1/K_d k_{tr}^{int}) \quad (7)$$

where the transfer rate k_{tr}^{int} due to internal catalysis in the open state is

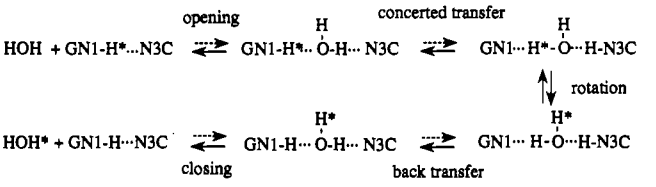
$$k_{tr}^{int} = R/(1 + 10^{\Delta pK}) \quad (8)$$

The constant R is a frequency that replaces the product $k_{coll}[C]$ of eq 3, and ΔpK is now the pK difference between GN1 and CN3 (or TN3 and AN1 for an A·T pair). The ΔpK values are so large (5 and 6, respectively, for A·T and G·C pairs) that transfer of the imino proton is generally inefficient and requires many opening events on the average. This is the reason why exchange may be accelerated by an external catalyst.

With more than one catalyst acting in the open state, the contributions to k_{tr} are additive. For the case of one added catalyst, together with the intrinsic catalyst, eq 2 becomes

$$\tau_{ex} = \tau_0 + K_d/(k_{tr}^{add} + k_{tr}^{int}) \quad (10)$$

Scheme I



Results

Symmetrical Chromomycin–d($A_2G_2C_2T_2$) Complex

As compared to the free duplex, imino proton exchange times and base-pair lifetimes are drastically increased in the region of the duplex interacting with the chromophore moieties of the chromomycin dimer.

Exchange in the Absence of Ammonia. The protons whose exchange is the slowest are the amino protons of G4 and the OH8 hydroxyl proton of chromomycin (Figure 1). Their exchange times, measured from spectra taken at different times after dilution in D_2O, are identical (240 min at 45 °C). The activation energy of the exchange time between 25 and 45 °C determined by the Arrhenius relation is 200 kJ/mol (Figure

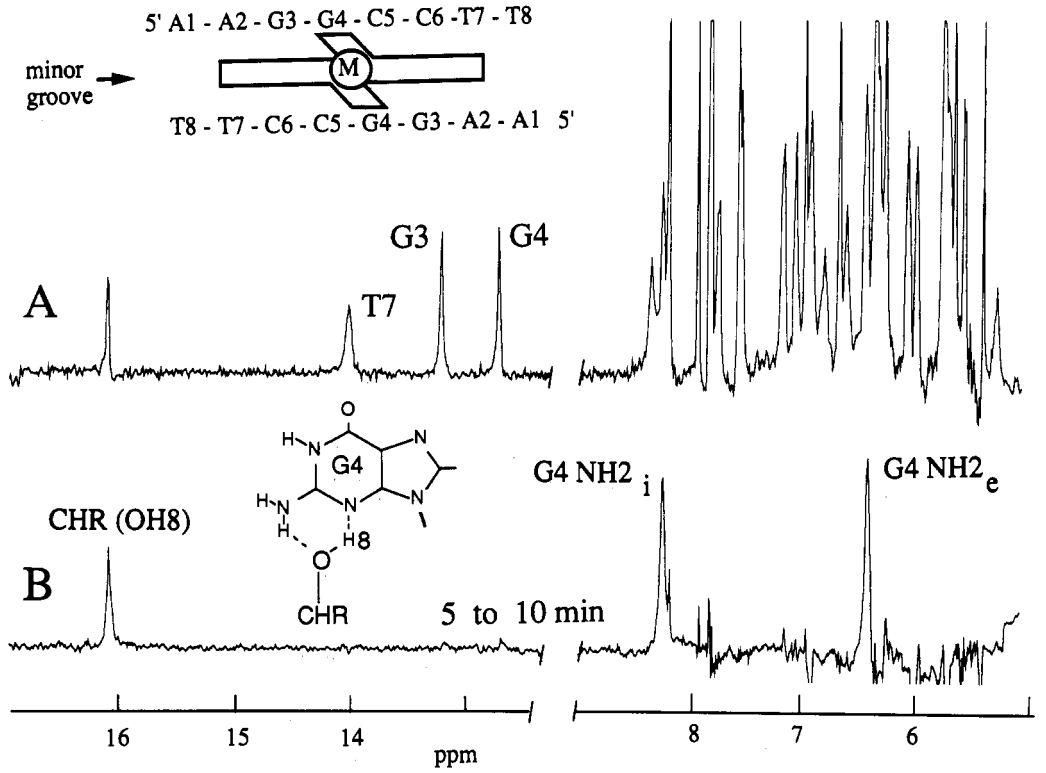


FIGURE 1: Real-time solvent exchange experiment in the symmetrical complex at 45 °C. (A) Reference spectrum in 90% H_2O. (B) Difference spectrum between two spectra obtained after transfer of a concentrated protonated sample in D_2O, the first spectrum being accumulated in the time interval indicated on the figure, the second spectrum during 30 min, starting 10 h after changing the solvent. The CHR (OH8) proton and the G4 amino protons exchange at the same rate. The exchange time, obtained from spectra recorded at different times after changing the solvent is 240 min. Experimental conditions: 0.1 M NaCl, pH 8.8.

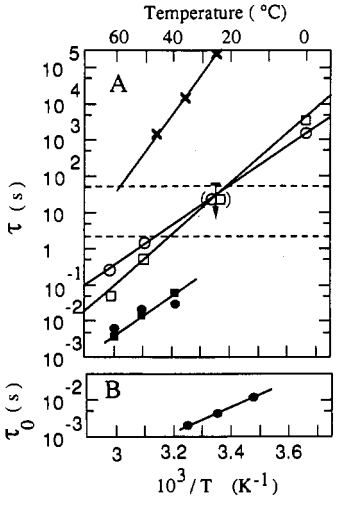


FIGURE 2: (A) Proton exchange times in the absence of added catalyst at pH 8.8 and base-pair lifetimes in the symmetrical d(A1-A2-G3-G4-C5-C6-T7-T8)–CHR complex versus the inverse of temperature. Open symbols are the exchange times of the G3 (□) and G4 (○) imino protons in the absence of added catalyst. The horizonal bar at the top of the arrow indicates the upper limit of the exchange time. The exchange times of G4 amino protons and of the CHR (OH8) proton are identical (X). Closed symbols are the base-pair lifetime of G3·C6 (■) and G4·C5 (●). Exchange times between 2 and 60 s (horizontal dotted lines) are inaccessible to NMR measurements. (B) The base-pair lifetimes of G4·C5 in the free $A_2G_2C_2T_2$ duplex.

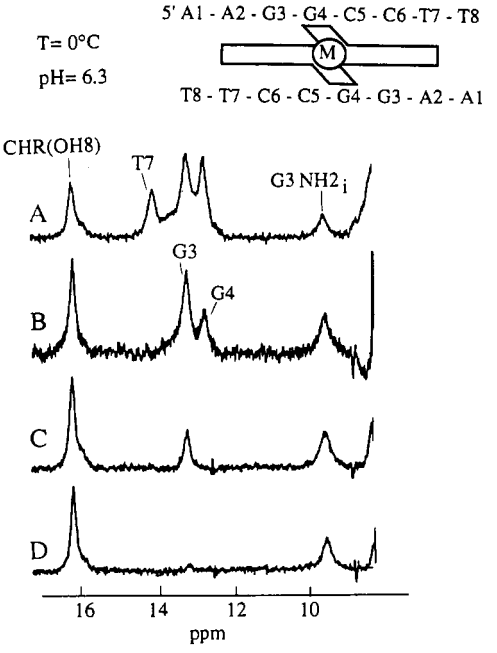


FIGURE 3: Real-time solvent exchange experiment in the symmetrical complex at 0 °C, pH 6.3. (A) Reference spectrum of the complex in 90% H_2O. Spectra B, C, and D were obtained, respectively, 4 min, 2.7 h, and 17.6 h after transfer of a concentrated protonated sample into D_2O. In spectrum D, the deuteration of the CHR (OH8) proton (16.1 ppm) and of the internal amino proton of G3 (9.5 ppm) is still negligible, indicating exchange times longer than 10 days. (The reference spectrum should be multiplied by 1.5 for normalization).

2A). At 0 °C, deuteration of the CHR (OH8) proton (16.1 ppm) and of the internal G amino proton of pair G3·C6 (9.5 ppm) is negligible up to 17 h after the solvent change (Figure 3), indicating exchange times longer than 10 days. No pH dependence was observed between pH 5 and 10.

The time course of imino proton exchange at 0 °C, pH 6.3, is shown in Figure 3. The exchange of the terminal A·T imino protons is too fast to be measured in real time experiments. The exchange times for the G3 and G4 imino protons are 3.2 and 227 min respectively.

The pH dependence of the G3 and G4 imino proton exchange times points to three different mechanisms (Figure 4).

Table I: Apparent Dissociation Constant (Given as $\alpha K_d \times 10^6$)[a] and Apparent Open-State Lifetime [Given in Italics as $\alpha\tau_{open}$ (ns)[b]] in the Free Duplexes and in Their Chromomycin Complex

d-(AAG-GCCTT)	free duplex (°C)				CHR complex (°C)[c]			
base pair	15	25	35	base pair	0	38	50	61
G3·C6	>10			G3·C6	0.015[d]	1.5	7.5	31
						78	*120*	*123*
G4·C5	1.5	3.6	22	G4·C5	7×10^{-4}[d]	0.15	0.3	0.4
	18	*14*	*44*			*4*	*6*	

d(AG-GATC-CT)	free duplex (°C)				CHR complex (°C)[c]			
base pair	10	20	30	base pair	0	30	40	50
G2·C7				G2·C7*	0.02[d]	0.6	1.5	7
						42	*22*	*49*
G3·C6	5	15		G3·C6*	2×10^{-3}[d]		0.4	
	60	*45*					*120*	
A4·T5	4	10	29	A4·T5*	0.1[d]	1	4	17
	≈20	*≈10*				*8*	*16*	*34*
				T5·A4*	0.015[d]	1	2.5	17.5
						≈4	*≈2*	
				C6·G3*	0.3[d]	6.7		
						27		

[a] The apparent dissociation constant αK_d is equal to the ratio of the imino proton exchange rate in the oligomer to that of the isolated monomer referred to the same low ammonia concentration, multiplied by the corrective factor of eq 3 (≈1.4). [b] The apparent lifetime of the open state $\alpha\tau_{open}$ is equal to $\tau_0 \times \alpha K_d$. [c] The stoechiometry is one Mg(II) and two chromomycins per duplex. [d] These dissociation constants are obtained from exchange catalysis by OH^-. Other dissociation constants are obtained from exchange catalysis by NH_3.

An acid-catalyzed process dominates up to pH 6 for the imino proton of G3 and up to a surprisingly high pH value (pH 8.5) for the G4 imino proton. The exchange time reaches a maximum, τ_{AAC}, between pH 6 and 8 for G3 (225 min) and between pH 8.5 and 9.8 for G4 (28 min). These exchange times are 100 and 10 times longer than those commonly measured in G·C pairs of a free duplex (Leroy et al., 1988a, and unpublished results). At high pH, exchange is catalyzed by OH^-. Using the data of Figure 4 and the k_i values given above for exchange catalysis by OH^-, we compute apparent dissociation constants of 1.5×10^{-8} and 7×10^{-10} for base pairs G3·C6 and G4·C5, respectively.

The activation energies of the G3 and G4 imino proton exchange times at pH 8.8 (Figure 2) are respectively 136 and 106 kJ/mol.

Exchange Catalysis by Ammonia. The imino proton spectrum of the symmetrical complex at 50 °C in 90% H_2O, pH 5.4, is plotted in the left panel of Figure 5. The effects of pH and ammonia on the magnetization transfer rate from water to imino and to CHR (OH8) protons are shown in the central and right panels. Exchange catalysis by ammonia is more efficient for the imino proton of G3 (it exhibits a greater increase in line width and a faster magnetization transfer rate from water) than for the imino proton of G4. The exchange rate of the CHR (OH8) proton is too long, even at 50 °C, to be measured by the saturation transfer method.

The effect of temperature is displayed in Figure 6A,B. For comparison, Figure 6C presents a measurement of the exchange catalysis of the G4 imino proton in the free duplex of $d(A_2G_2C_2T_2)$. We could not measure the exchange time of the G3 imino proton in the free duplex. Its NMR signal, poorly resolved from that of G4, broadens and becomes unobservable as ammonia is added.

In the complex, as in the free duplex, the exchange times vary linearly with the inverse of the ammonia concentration. The base-pair lifetimes obtained by extrapolation to infinite ammonia concentration are plotted vs reciprocal temperature in Figure 2. The lifetime of G4·C5 is increased by a factor of 10 upon CHR binding. The lifetime of G3·C6, which is

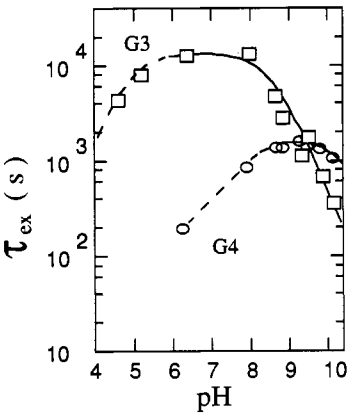


FIGURE 4: Exchange times vs pH at 0 °C of G3 (□) and G4 (○) imino protons in the symmetrical d(A1-A2-G3-G4-C5-C6-T7-T8)–CHR complex. At high pH, exchange is catalyzed by hydroxyl ions. Exchange in the pH-independent region is controlled by internal catalysis. The reduction of exchange times at low pH indicates an exchange process catalyzed by H^+. The solid curves are computed according to $\tau_{ex} = (1/\tau_{AAC} + k_i'[OH^-]\alpha K_d)^{-1}$ with the αK_d and τ_{AAC} values given in Tables I and II. The value of k_i' was obtained from eq 3 with the k_i value given in the text for catalysis by OH^-.

less than 1 ms in the free duplex, presumably due to fraying, becomes quite long in the complex. The activation energy of the G3·C6 and G4·C5 base-pair lifetimes (90 kJ/mol) is close to the value for G4·C5 in the $d(A_2G_2C_2T_2)$ free duplex (70 kJ/mol) and comparable to values (40 to 80 kJ/mol) commonly found in other free duplexes (Kochoyan et al., 1987, 1990; Leroy et al., 1988a).

The apparent base-pair dissociation constants computed from eq 5 with the data of Figure 6 and the k_i values given above are summarized in Table I.

Asymmetrical Chromomycin–d(AG$_2$ATC$_2$T) Complex

The 2-fold symmetry of the $d(AG_2ATC_2T)$ duplex is lost upon binding of the Mg(II)–CHR dimer (Gao & Patel, 1990). The two deoxynucleotide strands are therefore nonequivalent and will be distinguished by normal and starred lettering in the text below (see Figure 7). The two hydroxyl protons of

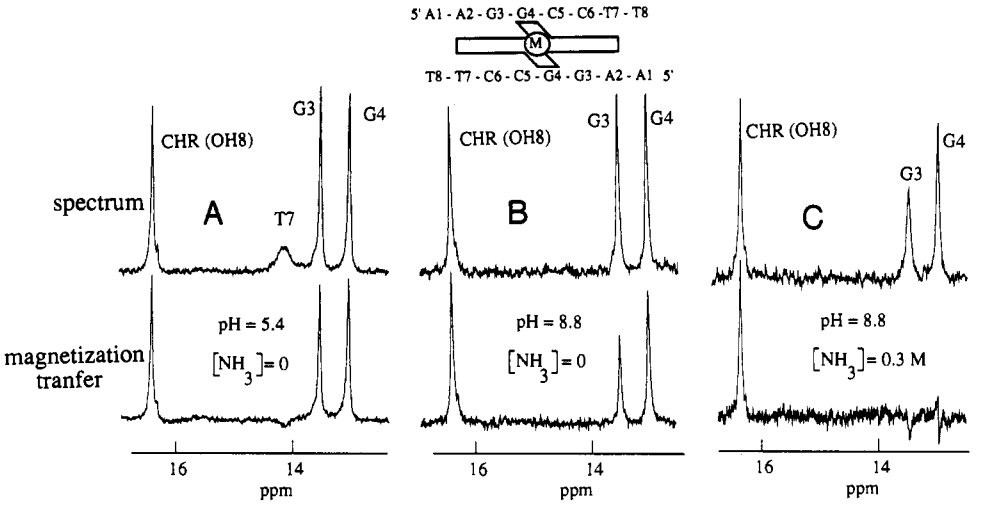


FIGURE 5: Effect of pH and ammonia on proton exchange in the symmetrical complex. The reference spectra (top) are obtained in the absence of ammonia at pH 5.4 (A), pH 8.8 (B), and in the presence of 0.3 M NH_3, pH 8.8 (C). The spectra below were obtained under the same conditions, 600 ms after selective inversion of the H_2O magnetization. The magnetization transfer was measured as a function of the time after selective inversion of water. From such data, the exchange times were computed according to Leroy et al. (1988a) (eq 5). Experimental conditions: 0.1 M NaCl, T = 50 °C.

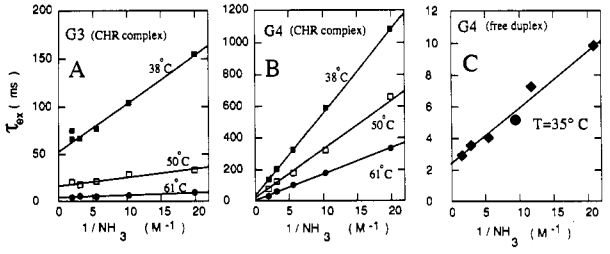


FIGURE 6: Exchange times of G3 (panel A) and G4 (panel B) imino protons in the symmetrical d(A1-A2-G3-G4-C5-C6-T7-T8)–CHR complex, at 38 °C, versus the inverse of the ammonia concentration. (Panel C) Exchange time of the G4 imino proton in the free duplex, at 35 °C. The exchange times vary linearly with the inverse of the ammonia concentration. The base-pair lifetimes obtained by extrapolation to infinite ammonia concentration are plotted on Figure 2, and the apparent dissociation constants obtained from the slope of τ_{ex} vs $1/[NH_3]$ are given in Table II.

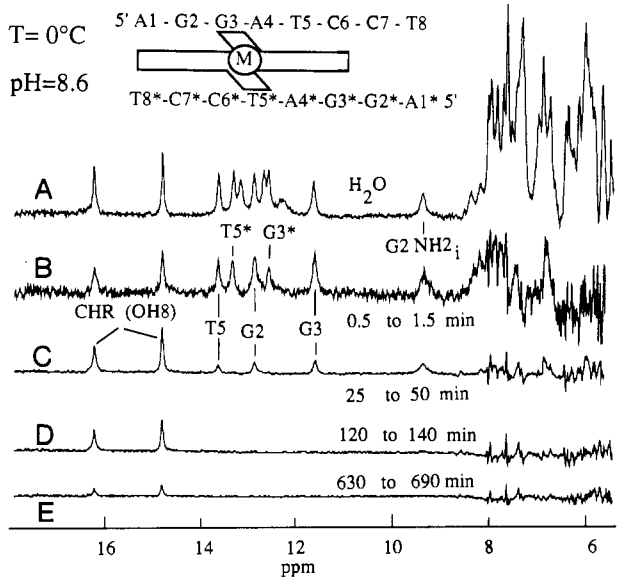


FIGURE 7: Real-time solvent exchange experiment in the asymmetrical complex at 0 °C. (A) Reference spectrum of the complex in 90% H_2O. The complex was diluted in D_2O at t = 0. The difference spectra B, C, D, and E are obtained by subtracting the spectrum recorded 24 h after transfer in D_2O, from those recorded in the time intervals indicated on the figure. The spectra are multiplied by a function that corrects for the amplitude response of the JR sequence (Guéron et al., 1991). Experimental conditions: 0.1 M NaCl, pH 8.6.

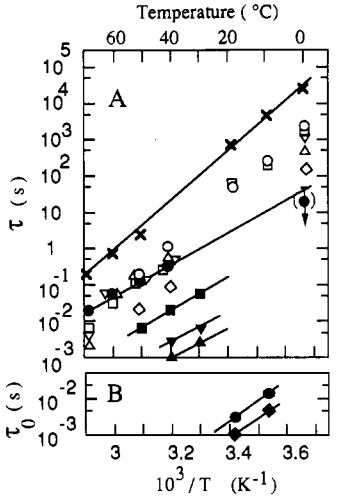


FIGURE 8: (Panel A) Proton exchange times in the absence of added catalyst and base-pair lifetimes in the asymmetrical d(A1-G2-G3-A4-T5-C6-C7-T8) complex versus the inverse of temperature. Open symbols are the imino proton exchange times in the absence of added catalyst at pH 8.8: G2 (□), G3 (○), T5* (△), T5 (▽), and G3* (◇). The exchange times of both CHR (OH8) protons (+, X) are identical and independent of added ammonia. Closed symbols are the base-pair lifetimes of G2·C7* (■), G3·C6* (●), A4·T5* (▲), and T5·A4* (▼). The lifetime (τ_0) of G3·C6* is particularly long; at 50 °C and above, it is comparable to the exchange time (γ_{AAC}) of the G3 imino proton, whose exchange is catalyst-insensitive in those conditions. (Panel B) Base-pair lifetimes in the free AG_2ATC_2T duplex: G3·C6 (●), A4·T5 (◆).

the CHR dimer are resolved in this complex. The 16.3 ppm hydroxyl proton is hydrogen-bonded to nitrogen N3 of G3, while the hydroxyl proton at 14.9 ppm is positioned in the minor groove close to the A4·T5* pair. In this complex also, imino proton exchange and base-pair opening are drastically slowed down.

Exchange in the Absence of Ammonia. The results of a real-time experiment carried out at 0 °C, pH 8.6, on the asymmetrical complex are shown in Figure 7. The two OH8 protons of the CHR dimer exchange at the same rate. The exchange time (8.3 h) and its activation energy (130 kJ/mol, Figure 8A) are much less than in the symmetrical complex (10 days and 200 kJ/mol). The exchange time of the internal G2 amino proton (9.4 ppm) is also shorter (40 min) than that

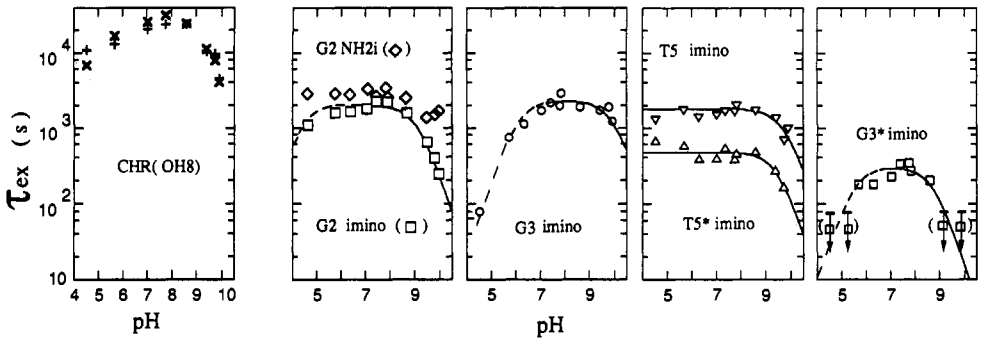


FIGURE 9: Exchange times vs pH in the asymmetrical d(A1-G2-G3-A4-T5-C6-C7-T8)–CHR complex at 0 °C: CHR (OH8) 16.3 ppm line (X), CHR (OH8) 14.9 ppm line (+), G2 (□), G3 (○), T5 (▽), T5* (△), and G3* (□) imino protons, G2 internal amino proton (◇). The horizontal bars at the top of the arrows in the right panel indicate upper limits for the exchange time of the G3* imino proton. The solid curves are computed according to the same procedure as in Figure 4.

of its counterpart (G3) in the symmetrical complex ($\tau_{ex} > 10$ days). Figure 7 shows that the amino protons of G3 (7.01 and 6.12 ppm) exchange much more rapidly than the CHR (OH8) protons, whereas those of their counterpart (G4) in the symmetrical complex exchange at the same rate as the CHR (OH8) protons. All the imino protons of the (-G2-G3-A4-T5-C6-)·(-C7*C6*T5*A4*G3*-) segment are still present 1 min after transfer from H_2O to D_2O (Figure 7), while imino proton exchange of T8, G2*, and T8* is too fast to be measured ($\tau_{ex} < 1$ min).

Figure 9 shows that the pH dependence of G2 and G3 imino proton exchange is comparable to that of their counterparts (respectively, G3 and G4) in the symmetrical complex. Imino proton exchange of G3* is also acid catalyzed. This is not the case for the T imino protons.

The activation energies for imino proton exchange in the absence of added catalyst (data of Figure 8A) are all about 150 ± 30 kJ/mol. The 50 °C imino proton spectrum is shown in Figure 10. In the absence of ammonia (Figure 10A), all the imino protons of the (-G2-G3-A4-T5-)·(-C7*C6*T5*A4*-)segment have comparable exchange times (from 230 ms for G3 to 180 ms for T5) while that of G3* is shorter: $\tau_{ex} \approx 13$ ms. The exchange times of both CHR (OH8) protons are identical: 1.5 s.

Exchange Catalysis by Ammonia. Ammonia has no effect on the exchange time of the CHR (OH8) protons. It accelerates exchange of all imino protons, except for the imino proton of G3 above 50 °C as can be seen by comparison of panels A and B of Figure 10. For the G3 proton, the longitudinal relaxation time decreases above 50 °C, an indication that it is dominated by the exchange contribution, and the exchange time is insensitive to hydroxyl (it is independent of pH) or ammonia. Hence, exchange is considered to be opening limited.

The base-pair lifetimes in the complex are displayed in Figure 8A. That of pair G3·C6* is particularly long (320 ms at 40 °C). At 0 °C the exchange times of all imino protons extrapolate to less than 0.8 min at infinite ammonia concentration, providing an upper limit for the base-pair lifetimes. For comparison, Figure 8B gives the base-pair lifetimes in the free duplex.

The apparent base-pair dissociation constants obtained for the asymmetrical complex are summarized in Table I.

Discussion

The first topic to be discussed is the kinetics of dissociation of the complexes. The information, interesting in itself, is required for the determination of base-pair kinetics in the complexes, the second topic, which will be discussed by reference to the isolated duplexes. The third topic is the process of imino proton exchange and its possible structural implications. The discussion is based on the symmetrical complex formed with the duplex of $d(A_2G_2C_2T_2)$, and we point out the particularities of the asymmetrical complex formed with the duplex of $d(AG_2ATC_2T)$.

Kinetics of Complex Dissociation

Chromomycin is nearly insoluble in water, but it is readily picked up by both oligomers in the form of a dimer of chromomycin associated with a divalent metal ion. The DNA–chromomycin complex does not form in the absence of divalent metal ions (Ward et al., 1965).

In the symmetrical complex, CHR (OH8) is doubly hydrogen bonded to G4 (Gao & Patel, 1990), as represented in Figure 1. The amino protons of G4 and of OH8 exchange at the same rate. Their exchange rate is unaffected by ammonia, and it is comparable to that of the metal chelated by the CHR dimer. For instance, the time constant of the exchange between free Zn(II) and Mg(II) in the complex is 61 h at 25 °C (Gao & Patel, 1990) whereas the exchange of the OH8 proton of CHR is 58 h (Figure 2A).

This suggests that exchange of the CHR hydroxyl proton is opening limited and requires dissociation of the DNA–drug complex. *We therefore equate the lifetime of the complex with the exchange time of CHR (OH8)*, plotted as crosses in Figure 2A. In this hypothesis, the large activation energy (200 kJ/mol) for exchange of the CHR hydroxyl and G4 amino proton then corresponds to the activation energy for complex dissociation.

The situation is the same in the asymmetrical complex, except for two differences. One is that the exchange rate of CHR (OH8) is now pH dependent (Figure 9). Rather than indicating that exchange is catalysis limited, this may correspond to a variation of the lifetime of the complex with pH. An argument in favor of this conclusion is that both (OH8) protons of the CHR dimer, although inequivalent in the asymmetrical complex (one is bound to G3 and the other is located close to the A4·T5* pair) have nearly the same exchange time in the entire pH range (Figure 9, leftmost panel). This time is therefore equated with the lifetime of the asymmetrical complex. It is plotted as crosses in Figure 8A and is hundreds of times shorter than that of the symmetrical complex (Figure 2A).

The other difference is that in the asymmetrical complex, the two amino protons of the guanine (G3), which is hydrogen bonded to CHR (OH8), exchange more rapidly ($\tau_{ex} < 20$ min at 0 °C) than the OH8 proton ($\tau_{ex} = 8.3$ h), thus indicating that G3 amino proton exchange occurs within the complex.

Figures 2 and 8 show that the lifetimes of both complexes are in all cases much longer than any imino proton exchange

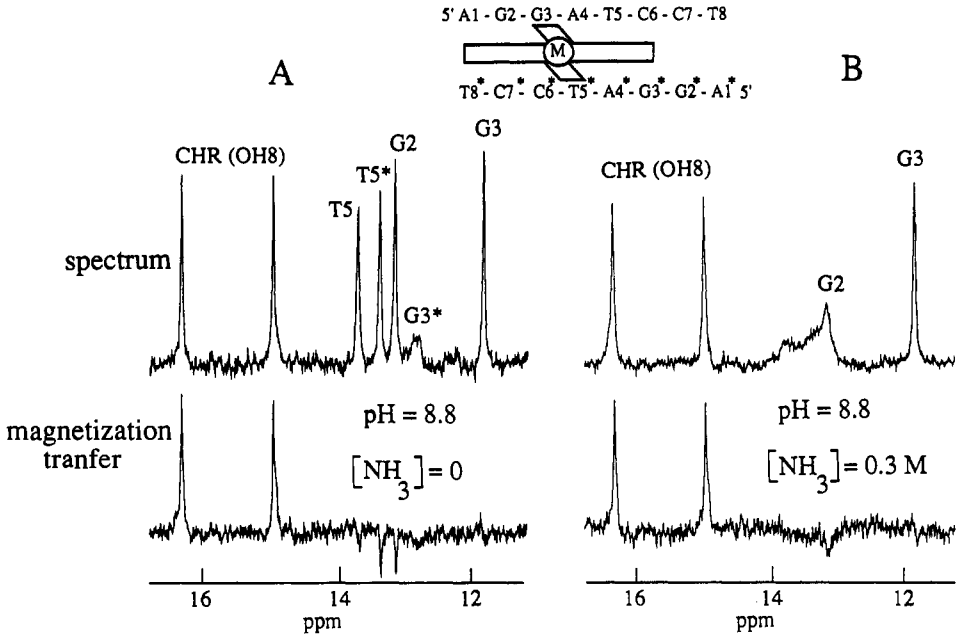


FIGURE 10: Effect of ammonia on the exchange broadening and on the magnetization transfer from H_2O in the asymmetrical d(A1-G2-G3-A4-T5-C6-C7-T8)–CHR complex. Top spectra: (A) Reference spectra obtained in the absence of ammonia and (B) in the presence of 0.3 M NH_3. The spectra below were obtained in the same conditions, 600 ms after selective inversion of the H_2O magnetization. Experimental conditions: 0.1 M NaCl, pH 8.8, T = 50 °C.

times. The exchange therefore takes place within the complex. Hence, base pairs can open in the complexed oligomer.

Base-Pair Kinetics

The largest effects of complexation concern base pairs in contact with the CHR chromophores (i.e., those of segments G3-G4-C5-C6 in the symmetrical complex). The lifetimes of these base pairs are considerably longer than those of the isolated duplexes (Figure 2). A clear case is that of pair G4·C5 whose lifetime is 2 ms at 35 °C in the isolated duplex and 29 ms in the symmetrical complex (Figure 2). The slowing down of base-pair opening by a drug in the minor groove does not necessarily mean that opening is toward this groove. In fact, one might guess that if this were the case, the opening should be inhibited much more than by a factor of 25. Another possibility is that opening is towards the major groove, but that it requires motion of the sugar–phosphate moieties into the minor groove and that this is sterically hindered by the presence of the drug. Alternatively, opening might require a movement toward the major groove that is inhibited by CHR (OH8), which holds back the G4 amino group into the minor groove, but this explanation is quite specific and does not tally with the observation that the opening of G3·C6 is also strongly slowed down.

In fact, the base-pair lifetime of G3·C6 in the complex is as long as that of G4·C5. This is quite different from the situation in the free duplex where G3·C6 has a very short lifetime, due most probably to fraying, since G3·C6 is separated from the end of the duplex by only two A·T base pairs. It thus seems that complexation to chromomycin may inhibit fraying. This could contribute to the inhibition of replication and of transcription by the drug. Despite the similarity in lifetime and activation energy for base-pair opening of these two base pairs, the value of the latter suggests independent opening. We did not detect any change in the base-pair lifetime of A2·T7, even though this base pair is in contact with the E saccharide ring of chromomycin in the minor groove (Gao & Patel, 1991).

Concerning the apparent base-pair dissociation constant αK_d (Table I), the four base pairs in contact with the chromophores

Table II: Proton Exchange Time (τ_{AAC}) and Rate of Intrinsic Catalysis in the Open State (k_{tr}^{int}) in the Absence of Added Catalyst, for the Chromomycin Complexes at 0 °C

d(A1-A2-G3-G4-C5-C6-T7-T8)-CHR			d(A1-G2-G3-A4-T5-C6-C7-T8)-CHR		
base	τ_{AAC} (min)	k_{tr}^{int} (s^{-1})[a]	base	τ_{AAC} (min)	k_{tr}^{int} (s^{-1})[a]
		imino protons			
T8	>1		T8*	>1	
T7	>1		G2	33	2.5×10^4
G3	225	4.9×10^3	G3	38	22×10^4
G4	28	8.5×10^5	T5*	7.5	2.2×10^4
			T5	30	3.7×10^4
			G3*	5	1.1×10^4
			G2*	>1	
			T8	>1	
		amino and CHR (OH8) protons			
G3 NH2i	>10 days		G2 NH2i	50	
G4 NH2i	>10 days		G3 NH2i	>20	
G4 NH2e	>10 days		G3 NH2e	>20	
CHR (OH8)	>10 days		CHR (OH8)[b]	415	

[a] k_{tr}^{int} is computed according to eq 8, by using the τ_0 and K_d values given in Table I. [b] The hydroxyl protons of the two chromomycin molecules of the asymmetrical complex exchange at the same rate.

of the CHR dimer are stabilized by one or two orders of magnitude in the complex. The stabilization of G4·C5 corresponds to both a longer base-pair lifetime and a shorter open state lifetime (Table I).

In the asymmetrical complex, independent opening of G2·C7* and G3·C6* is demonstrated by the different lifetimes. G3·C6* has a much longer lifetime than the other base pairs of the segment interacting with the CHR chromophores (Figure 8), and exchange of the G3 imino proton is opening limited at high temperatures. The lifetimes of A4·T5* and A5·T4* are shorter than those of the G·C pairs, although still much longer than in the free duplex (Figure 8).

The open state lifetime of G3·C6* is longer than that of the other base pairs of the (-G2-G3-A4-T5)·(-C7*C6*T5*A4*-) segment (Table I) and much longer (30 times) than that of its counterpart (G4·C7) in the symmetrical complex. This

gives rise to a situation of opening-limited exchange, as explained below.

Imino Proton Exchange

The Effect of Ammonia. In most cases, the exchange of imino protons in the complexes is catalyzed by ammonia. The phenomena are qualitatively similar to those observed previously in free duplexes (Kochoyan et al., 1987; Leroy et al., 1988a,b). However, the exchange times are much longer, corresponding to the reduction of the apparent dissociation constants αK_d with respect to free duplexes (Table I). If the value of α is one in the complexes, as it probably is in the free duplexes (Guéron et al., 1989), then the above statement applies to the true dissociation constants.

A particular case is that of the imino proton of G3 in the asymmetrical complex at high temperature: its exchange rate is not affected by catalysts, an indication that it is opening limited even without catalyst. This may be due to efficient intrinsic catalysis (as might happen for instance in the case of catalysis by a proton acceptor provided by the drug) and/or to a particularly long open-state lifetime. Indeed, according to eq 7, the opening-limited case is characterized by

$$\tau_0 \gg (1/K_d k_{tr}^{int}) \quad (10)$$

or

$$k_{tr}^{int}\tau_{open} \gg 1 \quad (11)$$

In fact, both effects may contribute, since $\alpha\tau_{open}$ (Table I) and k_{tr}^{int} (Table II) are both large for the G3·C6* pair at 0 °C.

Acid-Catalyzed Exchange. The exchange of the imino proton of guanosine is acid-catalyzed both in B- (Guéron et al., 1989) and in Z-DNA (Kochoyan et al., 1990). There is some evidence that this process involves protonation of cytidine at the N3 position and maybe transient Hoogsteen pairing. Indeed, G·C pairs in free duplexes of B-DNA switch from Watson–Crick to Hoogsteen pairing around pH 3, giving rise to a low-field signal from protonated cytidine (Guéron et al., 1989).

In the symmetrical complex, acid catalysis of exchange of the imino proton of G3 is comparable to that of the free duplex (data not shown). But in the case of G4, acid catalysis is so efficient that it is observed up to pH 8, instead of pH 5 in the free duplex. A downfield shift of the H8 proton of G4 is also observed at low pH (Gao & Patel, 1990). Both phenomena could be due to protonation at position N7, which would decrease the pK of the N1 group by 2 to 3 units (McConnell et al., 1978), thus enhancing intrinsic exchange catalysis of the imino proton (eq 6) and would also result in a downfield shift of the H8 proton (McConnell et al., 1983). For this explanation to hold, protonation at N7 would have to occur at a pH well above the normal pK of this group (pK = 2.1).

Another difference with the free duplex is that the low-field signal from Hoogsteen-paired protonated cytidine is not observed in the complex, even at 0 °C, pH 1.7 (data not shown).

Similar observations were made on the asymmetrical complex. Guanosine G3, which is expected to be H-bonded to chromomycin like G4 in the symmetrical complex (Figure 1), shows anomalous exchange catalysis by H^+. This phenomenon requires further investigation.

Structural Implications

In the symmetrical complex, the large changes in the kinetics of base-pairs G3·C6 and G4·C5 support the structure determined earlier by NMR (Gao & Patel, 1991), in which the chromophore component of the drug is bound by a pair of hydrogen bonds to G4. Both protons involved in this pair (the chromomycin OH8 proton and the external amino proton of G4) exchange only upon dissociation of the complex. So does the internal amino proton of G4, suggesting that there is no opening of the pair G4·C5 toward the minor groove in the complex.

The kinetic properties of the asymmetrical complex suggest the existence of interactions between the chromophore OH and G3, as expected if the coordination of the G2·C7* and G3·C6* pairs to the drug is the same in this complex as that of G3·C6 and G4·C5 in the symmetrical one. The faster exchange rate of the amino protons of G3 as compared to that of the chromophore OH suggests a process of partial opening of the connection between G3 and the chromophore OH8 in which the bond between O8 and the external amino proton of G3 would be broken, while that between the chromomycin H8 and the nitrogen of G3 would remain [the connection between G3 and CHR (OH8) in the asymmetrical complex is the same as that between G4 and CHR (OH8) in the symmetrical complex, which is shown in Figure 1]. The faster rate also indicates that the hydrogen bond between the internal amino proton of G3 and C7* can be disrupted within the asymmetrical complex.

The kinetic effects of the complexation on A4·T5* and T5·A4* are much smaller, indicating weaker interactions with the second chromomycin molecule. This is consistent with the much shorter lifetime of the asymmetrical complex, in which the interactions are strong with the G2-G3 segment and weak with the A4*-T5* segment, as compared with the symmetical complex, in which interactions are strong with the two equivalent G3-G4 segments.

In both complexes, the kinetics of other pairs are little affected by complexation, suggesting that the sugar components of the drug do not interact strongly with the oligomer, and that most of the free energy of binding may come from the interaction with the chromophores.

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Daunomycin Inverts the Long-Range Chirality of DNA Condensed States†

Carlos Bustamante,*,‡ Bruno Samorì,*,§ and Edoardo Builes‡

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, and Dipartimento di Chimica Organica, University of Bologna, viale Risorgimento 4, 40136 Bologna, Italy

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ABSTRACT: The effect of daunomycin upon DNA condensed states induced by poly(ethylene glycol) (PEG) was studied by circular dichroism (CD) and circular intensity differential scattering (CIDS). The CD spectra of these aggregates showed psi-type anomalies and intensities 10–100 times greater than those obtained with the dispersed DNA solutions in the absence of PEG. Increasing concentrations of daunomycin, added to the DNA solution prior to its aggregation, led, in the presence of PEG, to CD and CIDS signals which gradually decreased in magnitude and eventually inverted sign. The coincidence of the transition point of both signals and a careful characterization of the CD spectrum at the transition point clearly indicated that the inversion observed corresponds to an inversion of the handedness of the aggregates. The latter result suggests that the structure of the aggregates at the inversion point should resemble that of a nematic liquid-crystalline structure. The characteristic B-DNA spectrum obtained in this case further suggests that the packing process does not affect the secondary structure of the DNA molecules and that small changes in their local structure can induce dramatic changes in their long-range tertiary packing. The results obtained in this study represent a confirmation of a recent theory of psi-type CD in which the anomalous signals are interpreted as a manifestation of the long-range chirality of the aggregates.

In appropriate condensing agents, DNA molecules can condense and aggregate to form highly ordered structures which resemble in many ways those observed in vivo (Bouligand et al., 1968; Gourret, 1978; Brugerolles & Mignot, 1979; Livolant, 1984) and can be used as simplified models of the complex organization of DNA in eukaryotic chromosomes. These agents can be alcohols, such as ethanol (Reich et al., 1980; Huey & Mohr, 1981) and PEG (Lerman, 1971; Evdokimov et al., 1983; Evdokimov, 1988; Livolant & Maestre, 1988); H1 or H5 histones (Adler & Fasman, 1971; Sponar & Fric, 1972); polypeptides such as polylysine (Carrol, 1972; Shapiro et al., 1969; Haynes et al., 1970) and polyhistidine (Burchardt et al., 1973); divalent metallic ions (Simpson & Sober, 1970; Shin & Eichhorn, 1977); or lithium (Wolf et al., 1977).

The most obvious property displayed by these systems is the presence of anomalous circular dichroism (CD)[1] signals which are 10–1000 times larger than those of dispersed DNA. The shape of the spectrum is also greatly deformed by the presence of long tails extending toward the red wavelengths, outside the absorption bands of DNA. The anomalous spectra observed inside the absorption regions have been termed psi-type CD by Lerman et al. (1974), where "psi" stands for "polymer-and-salt-induced".

The liquid-crystal circular dichroism (LCCD) approach carried out by Saeva (1973, 1979) has been used to relate the anomalous signals to the presence of a long-range chiral organization in the DNA aggregates (Livolant & Maestre, 1988; Spada et al., 1988). However, this theory can describe only the optical activity of oriented cholesteric mesophases, and its extension to the treatment of rotationally averaged systems is by no means justified.

Recently, a general theory of CD of oriented and rotationally averaged systems has appeared in the literature (Keller & Bustamante, 1986a,b; Kim et al., 1986; Bustamante et al., 1988). This theory is valid for all dimensions of chiral order relative to the wavelength of light, and it reduces to the excitonic theories of Tinoco (1962) and De Voe (1965) in the limit of small chiral dimensions. This theory has shown that the anomalous psi-type signals are a manifestation of the long-range chiral structure in the DNA aggregates.

Evdokimov et al. (1983) have shown that complexes of DNA and certain drugs such as daunomycin, obtained in the presence of PEG, can give rise to psi-type signals of opposite sign to those obtained in the absence of the drug. The question then arises as to whether or not this sign inversion reflects an inversion in the handedness of the DNA aggregates induced by these drugs. In this paper, we present several lines of evidence, including CD, CIDS, and transmission electron microscopy (TEM), that the inversion in the sign of these

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* To whom correspondence should be addressed.
‡ University of New Mexico.
§ University of Bologna.

[1] Abbreviations: PEG, poly(ethylene glycol); CD, circular dichroism; CIDS, circular intensity differential scattering; LCCD, liquid-crystal circular dichroism; TEM, transmission electron microscopy; $I_{L,R}(\vartheta)$, intensities scattered at an angle ϑ for incident L and R circularly polarized light; r, moles of drug bound per mole of phosphate.

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