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Inhibition of the B to Z Transition in Poly(dGdC)·Poly(dGdC) by Covalent Attachment of Ethidium: Kinetic Studies†

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Received June 12, 1991; Revised Manuscript Received August 26, 1991

ABSTRACT: The photoaffinity analogue ethidium monoazide was used to prepare samples of poly(dGdC)·poly(dGdC) containing covalently attached ethidium. The effects of both noncovalently and covalently bound ethidium on the kinetics of the NaCl-induced B to Z transition in poly(dGdC)·poly(dGdC) was examined using absorbance and fluorescence spectroscopy to monitor the reaction. Covalently and noncovalently attached ethidium were equal in the extent to which they reduce the rate of the B to Z transition. By using fluorescence to selectively monitor the fate of noncovalently bound ethidium over the course of the transition, we found that ethidium completely dissociates as the reaction proceeds, but at a rate that lags behind the conversion of the polymer to the Z form. These experiments provide evidence for the redistribution of noncovalently bound ethidium over the course of the B to Z transition, leading to the development of biphasic reaction kinetics. The observed kinetics suggest that the primary effect of both covalently and noncovalently bound ethidium is on the nucleation step of the B to Z transition. The reduction in the rate of the B to Z transition by noncovalently or covalently bound ethidium may be quantitatively explained as resulting from the reduced probability of finding a drug-free length of helix long enough for nucleation to occur. As necessary ancillary experiments, the defined length deoxyoligonucleotides $(dGdC)_4$, $(dGdC)_5$, and $(dGdC)_6$ were synthesized and used in kinetic experiments designed to determine the nucleation length of the B to Z transition, which was found to be 6 bp. The activation energy of the B to Z transition was demonstrated to be independent of the amount of covalently bound ethidium and was found to be 21.2 ± 1.1 kcal mol^{-1}. Covalent attachment of ethidium was observed to increase the rate of the reverse Z to B transition, presumably by locking regions of the polymer into a right-handed conformation and thereby providing nucleation sites from which the Z to B conversion may propagate.

Left-handed Z-DNA, discovered by Pohl and Jovin (1972) and first visualized in the X-ray crystallographic studies from the Rich laboratory (Wang et al., 1979), is a dramatic example of DNA polymorphism. Interest in Z-DNA continues to be intense. Current understanding of the structure, dynamics, and function of Z-DNA has been summarized in several reviews (Rich et al., 1984; Soumpasis & Jovin, 1987; Jovin et al., 1987). The biological function of Z-DNA remains elusive, although Z-DNA has been demonstrated to exist in vivo in *Escherichia coli* (Jaworski et al., 1987), *Drosophila* (Lancillotte, 1987), and mammalian cells (Wittinger et al., 1989). Evidence has recently appeared for a regulatory role for Z-

† This work was supported by U.S. Public Health Service Grants CA-41474 (D.E.G.) and CA-35635 (J.B.C.), awarded by the National Cancer Institute, Department of Health and Human Services.
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0006-2960/91/0430-10931$02.50/0

DNA in gene expression (Jimenez-Ruiz et al., 1991).

Kinetic studies show that the NaCl-induced B to Z transition is slow, requiring 10^2–10^3 seconds to reach completion at ambient temperatures (Pohl & Jovin, 1972). Relaxation times for the transition are dependent on polymer length (Pohl & Jovin, 1972; Pohl, 1983; Walker & Abouela, 1988). Pohl and Jovin (1972) proposed that the B to Z transition proceeds by a rate-limiting nucleation step, followed by a rapid propagation of the transition along the DNA helix. Segments of Z-DNA may exist within oligonucleotides or restriction fragments flanked by B-DNA on either side, indicating that nucleation may occur within the DNA helix and need not begin from ends (Quadrifoglio et al., 1982; Klysik et al., 1981).

Small molecules (antibiotics, carcinogens, mutagens) may exert profound effects on the B to Z transition [reviewed in Rich et al. (1984)]. Pohl et al. (1972) first showed that the simple intercalator ethidium acts as an allosteric effector and converts Z-DNA to an intercalated, right-handed form. Equilibrium aspects of the allosteric conversion of Z-DNA to a right-handed form by a variety of intercalators has subsequently been studied in quantitative detail, which improves on the original work of Pohl et al. (1972) by using a more appropriate formalism for the DNA conformational transition and by including neighbor-exclusion effects (Chaires 1985, 1986b,c; Walker et al., 1985a,b; Britt et al., 1986; Lamos et al., 1986).

Kinetic studies of the effects of small molecules on the B to Z transition have been comparatively sparse. Pohl et al. (1972) utilized relaxation methods to study the mechanism by which ethidium acts as an allosteric effector on Z-DNA. Subsequent studies utilized transient kinetics to study the mechanism by which intercalators inhibit the B to Z transition. Mirau and Kearns (1983) and Chaires (1983) reported the effects of the intercalating drugs such a proflavin, ethidium bromide, actinomycin D, and daunomycin on the kinetics of the B to Z transition of poly(dGdC)·poly(dGdC). All of the drugs studied were shown to inhibit the rate of the B to Z transition. Mirau and Kearns (1983) concluded that the efficiency of inhibition correlates with the dissociation kinetics of the ligand. A revision of the Pohl and Jovin model was proposed in which nucleation of the left-handed conformation was not limited to the ends of the helix and either nucleation or propagation may be rate-limiting. Intercalating drugs, it was proposed, may inhibit either the nucleation or propagation steps, depending on their dissociation kinetics. According to this theory, drugs with slow dissociation kinetics should be more efficient inhibitors of the B to Z transition than those with relatively fast dissociation kinetics. However, this proposal does not appear to be the case for daunomycin, which has a dissociation rate of 1.2 s^{-1}, approximately 2 orders of magnitude slower than that of ethidium, yet both drugs demonstrate nearly identical effects on the transition rate (Chaires, 1983). Thus, although the dissociation kinetics of the drug–DNA interaction may play a vital role in the inhibition of the B to Z transition, it is clear that the mechanism for this inhibition remains poorly understood, and other factors must not be discounted.

We describe here kinetic studies that examine the effect of covalently and noncovalently attached ethidium on the NaCl-induced B to Z transition in poly(dGdC)·poly(dGdC). Our data provide considerable insight into the mechanism by which these intercalators inhibit the rate of the B to Z transition and show their primary effect is on the nucleation step. We find that inhibition of the B to Z transition by intercalators may be quantitatively correlated with a reduced probability of finding a long enough stretch of DNA free of drug suitable for nucleation.

Materials and Methods

Synthetic DNA Preparation. Poly(dGdC)·poly(dGdC) (Lot No. QF817910) was purchased from Pharmacia and used without further purification. The poly(dGdC)·poly(dGdC) was reported to have a sedimentation coefficient, $S_{20,w}$, equal to 5.6, corresponding to an average length of 249 base pairs. Stock DNA solutions were made by dissolving the solid sodium salts in 1 mL of 0.01 M sodium phosphate, pH 7.0/0.001 M disodium EDTA/0.01 M sodium chloride. Concentrations of polynucleotide solutions were determined by UV spectroscopy using the molar absorptivity value of 16 800 M^{-1} cm^{-1} for both polymers (Chaires, 1985).

Photoaffinity Probe. Ethidium monoazide, synthesized by the method of Graves et al. (1977), was dissolved in the low salt sodium phosphate buffer (described above), and the concentration was determined spectrophotometrically using a molar extinction coefficient of 5220 M^{-1} at 460 nm (Graves et al., 1977). All drug solutions were prepared immediately prior to the experiment.

Covalent Attachment of Ethidium Monoazide. Drug–DNA adducts were prepared by adding varying aliquots of the drug solution to a constant DNA solution under photographic safelight conditions. After equilibration at 5 °C for 60 min, the ethidum–DNA complexes were rendered irreversible via photolysis using two light boxes (Buchler Instruments) equipped with General Electric daylight No. F15T8-D light bulbs delivering energy at a rate of ca. 80 J m^{-2} s^{-1} (Graves et al., 1981). Photolysis was carried out on all samples simultaneously for 5 min at a constant temperature of 5 °C to ensure uniform adduct formation among the samples.

Drug that was not covalently attached to the DNA was subsequently removed using Chelex-100 (Bio-Rad). Small columns were prepared by packing a slurry of 5 mL of Chelex and water into a 10-mL syringe. Excess water was removed from the column by centrifugation at 700 rpm for 30 min. Samples were then pipetted to the top of the columns and eluted by centrifugation for 1 h at 1000 rpm. The temperature was maintained at 5 °C throughout the purification process.

The amount of ethidium bound to the DNA was quantitated by UV–visible absorbance at 500 nm using a molar extinction coefficient of 4100 M^{-1} cm^{-1} (Graves et al., 1981). DNA concentrations were obtained by monitoring the A_{260nm} absorbance after correction for the absorbance due to the bound drug.

Kinetic Studies of the B to Z Transition (Covalently Bound Ethidium). The kinetics of the B to Z transition of poly(dGdC)·poly(dGdC) following a sudden change in salt concentration were measured on a Varian Cary 2290 UV–visible spectrophotometer equipped with a Lauda constant-temperature circulating water bath. For the forward transition (B to Z), the change in absorbance at 295 nm was recorded as a function of time after the addition of 0.02 mL of concentrated poly(dGdC)·poly(dGdC) to 2.5 mL of 4 M NaCl phosphate buffer in a 1-cm path length UV cell to yield a final poly(dGDC)·poly(dGdC) concentration of 40 μM in base pairs (bp) (high salt jump). The absorbance was measured relative to a reference solution containing poly(dGdC)·poly(dGdC) in 2 M NaCl at 12-s intervals. The data were analyzed using EnzFitter (Elsevier-Biosoft) to a first-order rate equation providing the rate constant $k_{(B-Z)}$.

The reverse transition (Z to B) was accomplished by diluting a high salt sample of polynucleotide with low salt butter (low salt jump). The absorbance at 295 nm was recorded as a

function of time after the addition of 0.8 mL of 1.25×10^{-4} M (bp) poly(dGdC)·poly(dGdC) in 4 M NaCl to 1.7 mL of 0.01 M NaCl/sodium phosphate buffer. Because the Z to B transition occurs over a shorter time period than the B to Z transition, the absorbance was recorded at 3-s intervals relative to a reference solution containing poly(dGdC)·poly(dGdC) in 4 M NaCl. The final concentration of polynucleotides was 40 μM in base pairs, and the final salt concentration in the sample cell was 1.3 M NaCl. Data were analyzed as previously described, and the rate constant $k_{(Z-B)}$ was determined.

Kinetic Studies of the B to Z Transition (Noncovalently Bound Ethidium). The rate of the B to Z transition was monitored by absorbance measurements at 295 nm using a Perkin-Elmer λ 3 UV–vis spectrophotometer with data collected on a Bascom-Turner 3120 Electronic Recorder. Poly(dGdC)·poly(dGdC) at a concentration of 79 μM (bp) was equilibrated with ethidium under low salt conditions (0.2 M NaCl) at 20 °C. The B to Z transition was initiated by the addition of solid NaCl to give a final concentration of 4 M. The solution was rapidly mixed to dissolve the NaCl, and data acquisition began within 120 s. Absorbance was continuously monitored until constant absorbance was observed. In experiments designed to follow the fate of ethidium, the same protocol was followed except that the solution was divided into two aliquots following NaCl addition. The second aliquot was used for fluorescence detection, using a Perkin-Elmer 650-40 fluorescence spectrometer, with λ_{ex} at 484 nm and the λ_{em} at 595 nm. The experimental data from these absorbance or fluorescence experiments were digitized and fit appropriate kinetic models by nonlinear least-squares routines available as part of the NIH PROPHET computer resource, using methods previously described (Chaires et al., 1985).

Determination of the Nucleation Length of the B to Z Transition. Pohl and Jovin (1972) have shown that the nucleation length for the B to Z transiton may be obtained from measurements of the relaxation time for the transition as a function of polymer chain length. Under ionic conditions in which the degree of transition is independent of chain length, established by Pohl (1983) to be 2.25 M NaCl, the reciprocal relaxation time (τ^{-1}) is a function of chain length N as specified by the relation

$$\tau^{-1} = \left(\frac{k_f(\sigma_l - \sigma_r)}{2}\right)(N - \nu - 1)^{-1}$$

where ν is the nucleation length k_f is the propagation rate constant, and σ_l and σ_r are nucleation parameters (Pohl & Jovin, 1972). A plot of τ^{-1} versus $(N - \nu - 1)$ for oligonucleotides of different chain lengths results in a straight line through the origin if the nucleation length ν is properly chosen.

The deoxyoligonucleotides $(dGdC)_4$, $(dGdC)_5$, and $(dGdC)_6$ were synthesized by an Applied Biosystems Model 380 DNA synthesizer, deprotected with NH_4OH, and purified by acrylamide gel electrophoresis using standard procedures. Kinetics of the B to Z transition of these samples were determined by the absorbance method described above, with some modification. The reaction was initiated by the addition of microliter volumes of deoxyoligonucleotide solutions to 0.5 mL of 2.25 M NaCl equilibrated at 20 °C in the spectrophotometer cuvette. Data acquisition and analysis then followed the procedure described above.

Results and Analysis

Noncovalent Ethidium Interaction. The effect of noncovalently bound ethidium on the rate of the B to Z transition is shown in Figure 1. In order to facilitate comparison of data

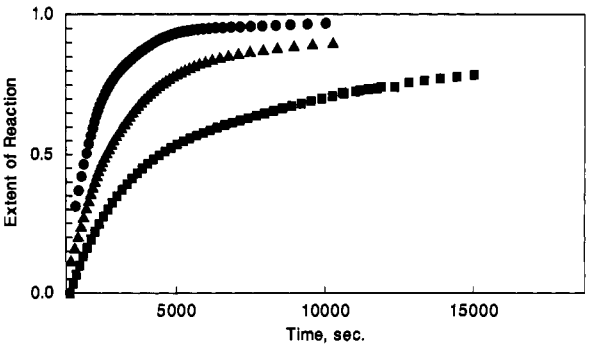

FIGURE 1: Time course of the B to Z transition of poly(dGdC)·poly(dGdC) in the presence and absence of noncovalently bound ethidium. The extent of reaction as monitored by absorbance measurements at 295 nm following a NaCl concentration jump to 4 M is shown as a function of time. The solid circles refer to 79.0 μM poly(dGdC)·poly(dGdC) in the absence of ethidium. The solid triangles refer to the same concentration of poly(dGdC)·poly(dGdC) plus 7.2 μM noncovalently bound ethidium (r_b = 0.092). The solid squares are for the identical solution but with fluorescence detection to monitor the fate of the ethidium over the course of the transition.

Table I: Rates of the B to Z Transition in Poly(dGdC)·Poly(dGdC) in the Presence of Noncovalently Bound Ethidium[a]

	absorbance		fluorescence
r_B	k_1 (s^{-1}) ($\times 10^3$)	k_2 (s^{-1}) ($\times 10^3$)	k_d (s^{-1}) ($\times 10^3$)
0.025	2.2	0.3	0.4
0.044	1.8	0.2	0.2
0.092	1.1	0.2	0.1

[a] Rates were determined by nonlinear least-squares fits of primary kinetic data. Two exponentials were required to fit absorbance data, while a single exponential adequately described the fluorescence data. Uncertainty in these estimates is 10–15%.

obtained by different optical methods, data are presented in Figure 1 as the extent of reaction, defined as $\theta = (S - S_0)/(S_\infty - S_0)$, where S_0 and S_∞ are the initial time and infinite time signals, respectively, and S is the observed signal at time t. Following a salt jump to 4 M NaCl, poly(GdC)·poly(dGdC) slowly converts to the Z conformation, with an apparent first-order rate constant of 3.3×10^{-3} s^{-1}. A single exponential adequately describes the time course in the absence of ethidium. In the presence of ethidium, two effects are evident. First, the rate of the transition is slowed (Figure 1). Second, the reaction becomes more complex, and two exponentials are required to describe the time course. Table I summarizes the estimates for the rates of the B to Z transition at several values of initially bound ethidium. For all concentrations of ethidium, the extent of the reaction is the same as that observed for poly(dGdC)·poly(dGdC) alone, and the polymer is fully converted to the Z conformation, although at a reduced rate.

The fate of the initially bound ethidium may be selectively monitored by fluorescence measurements. Dissociation of ethidium results in decreased fluorescence emission (λ_{em} at 595 nm) as illustrated in Figure 1. Ethidium dissociation over the course of the B to Z transition is adequately described by a single exponential, with a rate constant corresponding in magnitude to the slower phase observed by absorbance detection (Table I). The release of bound ethidium is shown to lag behind the initial rate of the B to Z transition. This fact is more clearly illustrated in Figure 2, which shows the extent of the reaction as monitored by fluorescence plotted as a function of the extent of reaction as monitored by absorbance. If ethidium dissociation was coincident with the B to Z transition, the extents of the reaction should be identical when monitored by the two optical methods, and data should fall

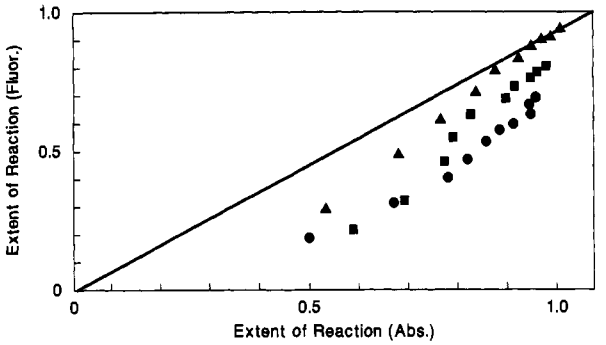

FIGURE 2: Comparison of the extent of reaction of poly(dGdC)·poly(dGdC)–ethidium complexes monitored by absorbance (Abs.) and fluorescence (Fluor). The symbols are for different binding ratios: ▲, 0.222; ■, 0.044; ●, 0.092. The solid line is the expected behavior if identical rates were monitored by the two optical signals. The solid line is the expected behavior if identical rates were monitored by the two optical signals. That the data do not follow this line indicates that ethidium dissociation, as monitored by the fluorescence signal, lags behind the B to Z transition, as monitored by the absorbance signal.

on the line shown in Figure 2. The observed data at three different initial binding ratios fall below this line, indicating that eithidium dissociation is slower than the B to Z transition, especially during the initial phase of the reaction. These data demonstrate that the greater the initial binding ratio, the slower is the relative dissociation compared to the B to Z transition.

Observations that ethidium dissociation lags behind the B to Z transition and that the kinetics of the transition are biphasic are consistent with a mechanism in which ethidium molecules redistribute into regions of the polymer remaining in the right-handed form as the reaction proceeds. The local binding ratios in such regions thus steadily increase over the course of the reaction, giving rise to the biphasic kinetics observed by absorbance detection. Such a kinetic mechanism is entirely analogous to and consistent with the mechanism proposed to explain the effect of intercalators on the thermally driven B to Z transition previously described (Chaires, 1986a).

The effect of covalently attached ethidium on the kinetics of the B to Z transition is shown in Figure 3. Covalently attached ethidium cannot dissociate as poly(dGdC)·poly(dGdC) converts from the B to the Z form. In contrast to the behavior observed for noncovalently bound ethidium, the time courses of the transitions shown in Figure 3 are in all cases adequately described by a single exponential. The rate constants obtained are tabulated in Table II. The extent of the reaction of poly(dGdC)·poly(dGdC) decreases with increasing

Table II: Rate Constants for the Forward B–Z Transition of Poly(dGdC)·Poly(dGdC) as a Function of the Amount of Covalently Bound Ethidium (Refers to 4 M NaCl Conditions)

	temperature (°C)	k_{BZ} (s^{-1}) ($\times 10^2$)	E_a (kcal/mol)
$r = 0.00$	10	0.91	21.1
	15	1.57	
	20	3.15	
	25	6.28	
	30	9.75	
$r = 0.03$	10	0.50	22.2
	15	0.96	
	20	1.90	
	25	3.65	
	30	6.42	
$r = 0.05$	10	0.51	22.0
	15	0.73	
	20	1.64	
	25	2.91	
	30	4.76	
$r = 0.14$	10	0.37	20.1
	15	0.58	
	20	1.19	
	25	1.65	
	30	3.41	

amounts of covalently bound ethidium, in contrast to the behavior observed for noncovalent ethidium (Figure 1).

A comparison of the relative rates of the B to Z transition with increasing binding ratios of both covalently and noncovalently bound ethidium is illustrated in Figure 4. This figure shows, unexpectedly, that covalently and noncovalently bound ethidium exert the same effect on the rate of the B to Z transition, providing considerable insight into the underlying mechanism by which the transition is inhibited. The B to Z transition is thought to proceed by a rate-limiting nucleation step, followed by propagation from the nucleation sites (Pohl & Jovin, 1972). Intercalation might inhibit either the nucleation or the propagation steps (Mirau & Kearns, 1983). The data of Figure 4 are quantitatively (at least up to one ethidium bound per 10 base pairs) consistent with a mechanism in which the primary effect of ethidium is to reduce the probability of nucleation of the B to Z transition and thereby slow the overall rate of the transition. Nucleation of the B to Z transition requires the creation of a stable nucleation site which is ν base pairs in length. Estimates for the nucleation length indicate that ν is equal to approximately 5 base pairs (Pohl & Jovin, 1972). In Figure 5, we provide an independent experimental determination of the nucleation length, using synthetic deoxyoligonucleotides of precisely defined length,

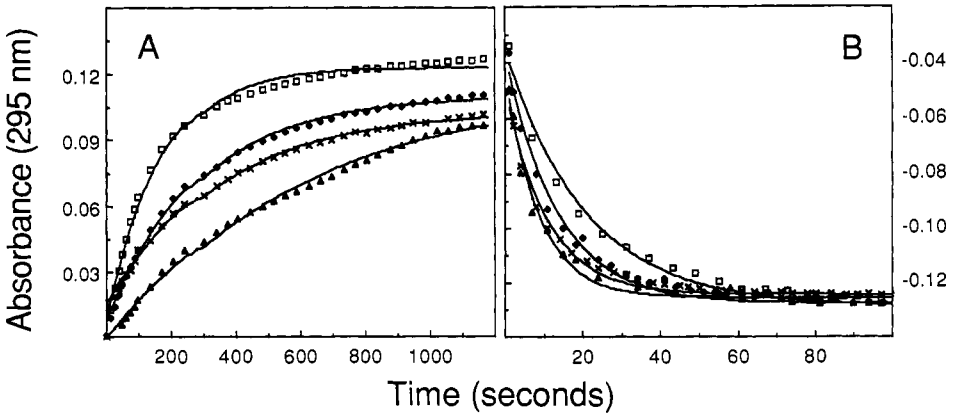

FIGURE 3: Representative absorbance traces for the B to Z (A) and the Z to B (B) transitions as a function of the amount of covalently bound ethidium. For both panels, the open squares (□) represent poly(dGdC)·poly(dGdC) with no drug bound. The remaining symbols refer to the following binding ratios (moles of ethidium per moles of base pairs): closed diamonds (◆), 0.03; (×), 0.05; closed triangle (▲), 0.142. The solid lines drawn through the data represent the best fit to a simple first-order rate equation.

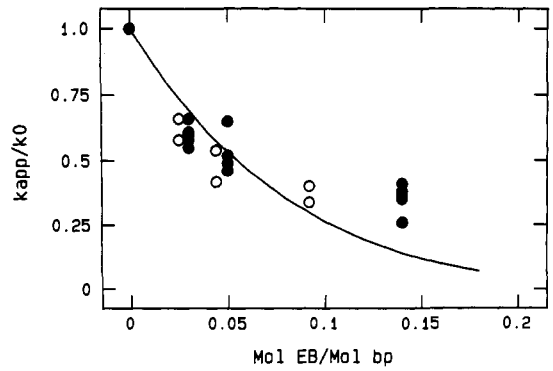

FIGURE 4: Summary of the relative rate of the B to Z transition as a function of noncovalently bound (○) and covalently bound (●) ethidium. The values of k_1 from Table I were used in the case of noncovalently bound drug. The solid line is the calculated probability of finding an ethidium-free length of 6 base pairs along the DNA lattice, assuming an exclusion parameter of 2 bp. See text for details.

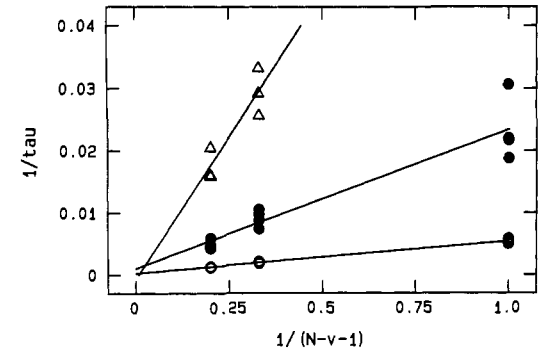

FIGURE 5: Experimental determination of the nucleation length of the NaCl induced B to Z transition in poly(dGdC)·poly(dGdC). The reciprocal relaxation time is plotted as a function of oligonucleotide length (N) and the nucleation length (ν). If the correct nucleation length is used, the data should be linear and pass exactly through the origin. Rates for the B to Z transition were determined at 12 °C (○), 20 °C (●), and 30 °C (△) using synthetic deoxyoligonucleotides 8, 10, and 12 bp in length. From these data, a nucleation length (ν) of 6 bp is obtained for the B to Z transition.

and determine the value of ν to be approximately 6 base pairs in length. Thus, in order for nucleation to occur in the presence of bound drug, a stretch of helix (free of drug) of at least 6 base pairs must exist. The probability of finding a stretch of helix ν base pairs long without bound ethidium is given by

$$\text{probability} = (1 - nr)^{\nu}$$

where n is the exclusion parameter and r is the binding density. The solid line in Figure 4 was calculated by assuming n equal to 2 and ν equal to 6. This calculation indicates that the reduction in the relative rate of the B to Z transition upon binding of ethidium (either covalently or noncovalently) may be quantitatively accounted for by the decreased probability of finding a length of helix not containing ligand sufficiently long for nucleation. We note that data above $r = 0.1$ clearly deviates from the calculated curve. This may reflect an additional effect of bound intercalator on the propagation step of the B to Z transition at these high binding ratios.

The effect of covalently bound ethidium on the reverse Z to B reaction was also examined (Figure 3B). In this case, covalently attached ethidium should increase the rate, since the ligand should lock the helix into a right-handed conformation that would serve as a nucleation site. Such an effect is in fact observed. Figure 3B shows the time courses of the reverse Z to B transition, which are in all cases adequately described by a single exponential. The rate constants for this transition as a function of ethidium binding density are collected in Table III.

Table III: Rate Constants for the Reverse Z–B Transition of Poly(dGdC)·Poly(dGdC) as a Function of the Amount of Covalently Bound Ethidium (Refers to 1.3 M NaCl Conditions)

	temperature (°C)	k_{BZ} (sec^{-1}) ($\times 10^2$)	E_a (kcal/mol)
$r = 0$	10	1.36	18.2
	15	1.73	
	20	3.47	
	25	4.82	
	30	11.60	
$r = 0.03$	10	1.40	21.1
	15	2.99	
	20	4.92	
	25	9.35	
	30	17.10	
$r = 0.05$	10	2.02	20.1
	15	3.02	
	20	5.56	
	25	9.83	
	30	20.80	
$r = 0.14$	10	2.65	18.8
	15	6.65	
	20	8.49	
	25	17.90	
	30	23.80	

Experiments were designed to monitor the kinetics of the B to Z transition over several different temperatures and drug concentrations. From the rate constants obtained (Table II), the activation energy, $E_{a(B-Z)}$, was calculated using the Arrhenius equation

$$\ln k = \left(\frac{-E_a}{RT}\right) + \ln A$$

where k is the rate constant, E_a is the activation energy, and A is the Arrhenius frequency factor. A graph of ln k versus $1/T$ results in a linear plot with a slope equal to $-E_a/R$ (data not shown). The rate of the B to Z transition of ethidium-modified poly(dGdC)·poly(dGdC) decreases as the concentration of covalently attached drug is increased, as summarized in Table II. The activation energies of the B to Z transition calculated from the slopes of the lines were 21.2 ± 1.1 kcal/mol for all ethidium–poly(dGdC)·poly(dGdC) adducts, indicating that although covalent attachment of ethidium to poly(dGdC)·poly(dGdC) resulted in a dramatic change in the transition kinetics, it had no effect on the activation energy for the B to Z transition. Using the data shown in Figure 5, estimates for the activation energies for the B to Z transition of oligonucleotides 8 to 12 bp long were obtained. Within error, the activation energy was the same for these samples, with E_a equal to 23.6 ± 3.5 kcal/mol. That the activation energy for these short oligonucleotides is essentially the same as the value for poly(dGdC)·poly(dGdC) confirms that the energetics of the transition is independent of chain length and reinforces that the large magnitude of E_a may by attributed to the nucleation reaction.

The kinetics of the reverse transition (Z to B) were also measured as a function of temperature (Table III). Rate constants for the Z to B transition are much faster than the corresponding rates for the B to Z transition and increase as the concentration of covalently bound ethidium was increased. Arrhenius plots were used to calculate the energy of activation, $E_{a(Z-B)}$ for the Z to B transition. The activation energy of this transition was also essentially constant, both for the free poly(dGdC)·poly(dGdC) and the ethidium-modified poly(dGdC)·poly(dGdC) at 19.8 ± 1.6 kcal/mol, indicating that the covalent attachment of ethidium did not influence the

activation energy of the Z to B transition.

Discussion

The experimental results described here show that noncovalently and covalently bound ethidium slow the rate of the B to Z transition in poly(dGdC)·poly(dGdC) to the same extent. There are, however, distinct differences between the effects of noncovalently and covalently attached ethidium on the time course of the reaction. First, the overall extent of the reaction is different in two cases. Fluorescence measurements designed to selectively monitor the fate of noncovalently attached ethidium show that eventually the drug is completely dissociated over the course of the B to Z transition, at concentrations used in these studies. Poly(dGdC)·poly(dGdC) in this case is completely converted to the Z form, at all ethidium concentrations examined. In contrast, covalently attached ethidium cannot dissociate, and a decrease in both the rate and the overall extent of the reaction with increasing binding ratios is observed. The second difference is in the complexity of the time course of the B to Z transition observed in the presence of noncovalently or covalently bound ethidium. In the case of noncovalently attached ethidium, the time course becomes biphasic in the presence of ligand. In contrast, the time course of the covalently attached ethidium reaction remains monophasic. These kinetic observations provide considerable new insight into the mechanism by which ethidium inhibits the B to Z transition.

Pohl and Jovin (1972) proposed that the B to Z transition proceeds by a rate-limiting nucleation step, followed by propagation along the helix from the nucleation sites. Both the rates and the activation energies we observe for poly(dGdC)·poly(dGdC) in the absence of ethidium in the studies described here (Tables I–III) are in excellent agreement with the original values reported by Pohl and Jovin (1972). Mirau and Kearns (1983) pointed out that intercalators may slow the B to Z transition by altering either the rate of nucleation or propagation and concluded that different intercalators exert their effect on different steps, arguing that the drug dissociation constant was the primary determinant of how effectively an intercalator would inhibit the B to Z transition, with those compounds with slow dissociation rates being more effective inhibitors. The results we obtain here vitiate this latter point, since covalently attached ethidium will not dissociate at all yet is identical to noncovalently attached ethidium in its effectiveness as an inhibitor of the B to Z transition. Our data suggest that the primary effect of both noncovalently and covalently attached ethidium is on the nucleation step. Below we will show that reconsideration of the data of Mirau and Kearns (1983) obtained with three other intercalators is consistent with this conclusion.

Figure 4 shows that the relative decrease in the rate of the B to Z transition with increasing binding ratios of ethidium is directly correlated to the decrease in the probability of finding a length of helix containing no drug that is long enough for nucleation. We have experimentally determined the nucleation length to be 6 bp (Figure 5). The probability of finding a length of helix without bound ligand is $P = (1 - nr)^6$, where n is the exclusion parameter and r is the binding ratio (McGhee & von Hippel, 1974). In their 1983 analysis, Mirau and Kearns incorrectly specified that probability as $(1 - r)^{-1}$, neglecting the effect of perhaps the key factor that determines the effectiveness of an intercalator as an inhibitor of the B to Z transition, the exclusion parameter. The exclusion parameter controls the probability of finding a length of helix long enough for nucleation. At identical binding ratios r, compounds with larger exclusion parameters will more effectively inhibit the B to Z transition because the probability of nucleation will be less. Reexamination of the data of Mirau and Kearns (1983) reinforces this point. Table IV shows the original data of Mirau and Kearns, exclusion parameters for the compounds they studied, and the expected ratios for the probability of nucleation. The ratio of the observed rate relative to ethidium (k/k_{eb}) correlates well with the calculated ratio (P/P_{eb}) of finding an appropriate nucleation site. The remarkable effectiveness of actinomycin is seen by this calculation to be a direct consequence of its large exclusion parameter (6 bp). The agreement of these data with our proposed model is excellent, given that Mirau and Kearns specified only the ratio of added intercalator to base pair and not the binding ratio r that we need for our calculation. The latter quantity will differ from the former for each of the compounds listed because of the differences in the binding constants. Table IV leads us to reinterpret the data of Mirau and Kearns and conclude, in agreement with our studies, that the predominant effect of intercalators on the B to Z transition arises from a reduction in the probability of the nucleation step.

Table IV: Reevaluation of the Data of Mirau and Kearns[a]

sample	k (s^{-1}) ($\times 10^3$)	k/k_{eb}[b]	n (bp)	P/P_{eb}[c]
poly(dGdC)	7.4			
+ethidium	4.5	1.0	2.0	1.0
+proflavin	5.0	1.1	2.0	1.0
+bismethyidiumspermine	0.98	0.2	4.0	0.18
+actinomycin D	<0.1	0.02	6.0	0.015

[a] Data were taken from Mirau and Kearns (1983). Final solution conditions: 3.5 M NaCl, 34 °C, with 0.1 mol of drug/bp *added*. [b] The ratio of the observed rate to that of ethidium is shown as the ratio k/k_{eb}. [c] The ratio P/P_{eb} is the calculated ratio of the probabilities of finding a drug-free length of helix 6 bp long (the value of the nucleation length), where P_{eb} is the probability for ethidium. The general equation for the probability is $P = (1 - nr)^6$ where n is the exclusion parameter and $r = 0.1$. The listed values for n were obtained from the literature as follows: ethidium (Walker et al., 1986), proflavin (Chaires, 1986); bismethidiumspermine (Dervan & Becker, 1978); actinomycin D (Dougherty & Pigram, 1982).

Pohl and Jovin (1972) found that the kinetics of the B to Z transition were length dependent but that the transition activation energy was not, an observation we have confirmed using deoxyoligonucleotides of defined length. They proposed that the slow step in the transition and the high activation energy resulted from the nucleation of a stretch of Z-DNA with the B helix. Our results support this conclusion. Increasing the amount of covalently attached ethidium should not affect the activation energy if the primary effect is on the nucleation step as we have observed. Covalent ethidium attachment should increase the rate of the reverse transition because the right-handed drug–DNA adduct site provides an existing nucleation site for the B conformation.

Following nucleation, the transition of the helix from the B to Z form is propagated from nucleation sites, at rates perhaps as high as 10^7 s^{-1} (Pohl & Jovin, 1972). The lifetime of the drug–DNA complex should be a crucial determinant of how effectively the drug will inhibit the propagation step, with long-lived complexes interfering to a greater extent. Our results suggest that covalently attached ethidium inhibits propagation in addition to its primary effect on nucleation. A manifestation of such inhibition is that increasing levels of covalently bound ethidium decrease the overall extent of the reaction. Covalently bound ethidium molecules lock stretches of the helix into the right-handed conformation and interrupt propagation. The case of noncovalently bound ethidium is more complicated. The B to Z transition becomes kinetically more complex in the presence of noncovalently bound ethi-

dium. The reaction proceeds to completion, however, although at a slower rate. By selectively monitoring the fate of ethidium over the time course of the B to Z transition, we demonstrate (Figures 1 and 2) that ethidium is eventually completely dissociated, although the rate of the dissociation lags behind the rate of the B to Z transition. The following mechanism can account for these observations. Following nucleation, propagation proceeds with little hindrance from noncovalently bound ethidium, since the lifetime of the ethidium–DNA complex is short. Transiently dissociated ethidium will not remain free, however, but will bind to remaining regions of the helix in the right-handed conformation, producing regions of the helix with high local ethidium binding densities as the reaction proceeds. Nucleation in these regions becomes less probable as the local binding densities grow, leading to a slow kinetic phase. The key feature of the mechanism is the redistribution of ethidium over the course of the transition, which is directly responsible for the biphasic kinetics that develop in the presence of ethidium. The proposed mechanism is entirely analogous to the mechanism proposed to account for the effect of binding drugs on the helix to coil transition (Crothers, 1971; McGhee, 1976), which has been quantitatively applied to explain the effect of intercalators on the thermally driven B to Z transition in poly(dGm^{5}dC)·poly(dGm^{5}dC) (Chaires, 1986a).

In summary, we propose that the primary effect of intercalators is on the nucleation event leading to the B to Z transition. Since nucleation is itself a slow, improbable event, the lifetime of the intercalator–DNA complex should have little effect on this step. The binding density and the neighbor-exclusion parameter combine to govern the probability of finding a drug-free stretch of helix sufficiently long for nucleation to occur, a length we have experimentally determined to be 6 bp for the NaCl-induced B to Z transition in poly(dGdC)·poly(dGdC). Following nucleation, intercalators might additionally inhibit the propagation step if the drug–DNA complex lifetime is long compared to the rate of propagation. Regardless, intercalators may be expected to redistribute over the course of the B to Z transition, creating local right-handed regions with high binding densities, which lead to biphasic kinetics. Such effects have been demonstrated here in the case of noncovalently attached ethidium.

Registry No. Poly(dGdC), 36786-90-0; $(dGdC)_4$, 80458-01-1; $(dGdC)_5$, 76957-82-9; $(dGdC)_6$, 91308-73-5; ethidium, 3546-21-2; ethidium monoazide, 63783-82-4.

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