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

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ABSTRACT: The effects of covalent modification of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) by ethidium monoazide (a photoreactive analogue of ethidium) on the salt-induced B to Z transition are examined. Earlier studies have shown ethidium monoazide to bind DNA (in the absence of light) in a manner identical to that of the parent ethidium bromide. Photolysis of the ethidium monoazide–DNA complex with visible light results in the covalent attachment of the photoreactive analogue to the DNA. This ability to form a covalent adduct was utilized to probe the effects of an intercalating irreversibly bound adduct on the salt-induced B to Z transition of the poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) polynucleotides. In the absence of drug, the salt-induced transition from the B to Z structure occurs in a highly cooperative manner. In contrast, this cooperativity is diminished as the concentration of covalently attached drug is increased. The degree of inhibition of the B to Z transition is quantitated as a function of the concentration of covalently attached drug. At a concentration of one drug bound per four base pairs for poly(dGdC)·poly(dGdC) and seven base pairs for poly(dGm^{5}dC)·poly(dGm^{5}dC), total inhibition of this transition is achieved. Lower concentrations of bound drug were effective in the partial inhibition of this transition. The effects of the covalently bound intercalator on the energetics of the B to Z transition were determined and demonstrated that the adduct is effective in locking the alternating copolymer in a right-handed conformation under high salt conditions.

The conformational flexibility of DNA has been well-established over the last decade with the discoveries of left-handed DNA and most recently parallel strand DNA structures (van de Sande et al., 1988). Since the observation of the salt-induced transition of poly(dGdC)·poly(dGdC) from a right- to left-handed Z conformation by Pohl and Jovin (1972) and subsequent determination of the crystallographic structure by Wang et al. (1979), considerable interest has been generated concerning the biological relevance of left-handed DNA and its possible roles in gene regulation.

Z-DNA has been shown to be highly immunogenic, and antibodies have been produced and used to detect the occurrence of Z-DNA in vivo systems, such as polytene chromosomes from *Drosophila* (Pohl, 1983; Moeller et al., 1982; Thomae et al., 1983; Nordheim et al., 1981; Lancillotti et al., 1985) and *Chironomus* (Jovin et al., 1983; Arndt-Jovin et al., 1983). In 1982, Nordheim and co-workers isolated a class of proteins from *Drosophila* that bind specifically to Z-DNA. Regions of Z-DNA have been found in the transcriptional enhancer region of the simian DNA tumor virus (SV40) minichromosome (Nordheim et al., 1983), suggesting that Z–DNA may act as a positive regulatory transcription signal. The gradual removal of a segment with the potential of forming Z-DNA in an inactivated *Xenopus* Met-tRNA gene resulted in transcriptional reactivation (Hipskind & Clarkson, 1983), indicating that Z-DNA may also act as a negative regulatory signal decreasing the level of transcription. These findings indicate the natural occurrence of Z-DNA and its possible role in gene expression. However, the precise role of Z-DNA in vivo remains undefined.

From the observation of Z-DNA in natural DNA sequences, it is recognized that both right- and left-handed conformations may exist within the same segment of DNA. Early studies of the B–Z junction utilized systems in which a potential Z-forming sequence was cloned into a plasmid genome (Singleton et al., 1982; Johnston & Rich, 1985). More recently, solid-phase oligonucleotide synthesis was used by Sheardy (1988) to synthesize hexadecanucleotides designed with a specific base sequence such that under high salt conditions half of the duplex would assume a left-handed conformation while the other half would remain in the right-handed conformation. Experimental data suggest that the junction region between the Z and B DNA conformations possesses transient single-stranded character (as evidenced by reactivity to chemical probes specific for single-stranded DNA) or unpaired bases (Palecek et al., 1987; Kilpatrick et al., 1983; Stirdivant et al., 1982) and that the interface is probably small with only one base pair being dramatically distorted (Sheardy & Winkle, 1989; Pochet et al., 1986; Azorin et al., 1984). Recently, Peticolas and co-workers demonstrated by Raman spectroscopy that the length of the junction between B and Z conformations of DNA was less than three base pairs and may be independent of the base sequence of the junction region. The underwound nature of the junction region has been proposed to enhance reactivity of protein and drug binding which may affect gene expression.

Physical properties such as the structure and energetics of the B–Z junction govern the rate and extent of transition and may influence the functional roles of Z-DNA in vivo. However, little is known concerning the structural geometry and dynamics of the junction region. DNA binding drugs have been used to probe DNA structure and are known to affect the conformation of DNA and conversions between alternate conformations. In particular, intercalating drugs have dem-

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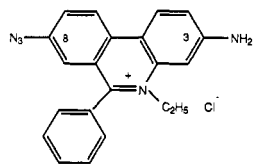

FIGURE 1: Chemical structure and numbering scheme of ethidium monoazide (8-azido-3-amino-6-phenyl-5-ethylphenanthradinium chloride).

onstrated profound effects on the Z conformation. One intercalator that has been widely studied and well-characterized for its effects on Z-DNA and the B–Z transition is ethidium bromide. The intercalation of ethidium was shown to induce a highly cooperative conversion of the poly(dGdC)·poly(dGdC) to a right-handed conformation at the high salt conditions (Pohl, et al., 1972). The equilibrium binding of ethidium to poly(dGdC)·poly(dGdC) under high salt conditions (4 M NaCl) exhibited positive cooperativity as indicated by the initial positive slope of the Scatchard plots (Walker et al., 1985). Kinetic studies have demonstrated that ethidium inhibits the B to Z transition of poly(dGdC)·poly(dGdC) (Mirau & Kearns, 1983). Inhibition of the transition was interpreted in terms of a two-step mechanism that includes a rate-limiting nucleation of the left-handed conformation followed by a rapid propagation of the transition down the DNA helix. The nucleation step was proposed to involve the unwinding or unstacking of one or more base pairs so that the guanine bases can adopt a syn conformation (Harvey, 1983). Complex formation by ethidium was thought to interfere with either the nucleation step at high binding ratios or the propagation step at low binding ratios of the B–Z transition of poly(dGdC)·poly(dGdC) (Mirau & Kearns, 1983).

Studies utilizing ethidium as a probe of DNA structure are limited by the reversible nature of ethidium–DNA complex. The bound drug is free to dissociate from the DNA helix, therefore limiting direct measurements of structural changes induced by complex formation. In an effort to probe this region and further characterize the structural and chemical properties associated with the B to Z transition, photoaffinity labeling is utilized. Photoaffinity labeling provides a means of rendering the noncovalent ethidium–DNA complex to be converted to an irreversible drug–DNA adduct. Ethidium monoazide (3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride) (shown in Figure 1) was chosen for these experiments due to its similarities in binding affinity and spectral properties to the parent ethidium bromide. Ethidium monoazide forms a noncovalent complex with DNA in the dark that is identical to the parent compound (Graves et al., 1981). Visible light is used to activate the azide moiety to a reactive nitrene intermediate which forms a covalent bond with the DNA. The use of ethidium monoazide as a drug probe provides a novel and useful approach for examining the B–Z transition properties. By examination of the salt-induced B–Z transition of these ethidium–DNA adducts, binding stoichiometries and energetics associated with the B to Z transition can be determined. These studies provide information on the effects of drug binding on the structural properties of Z-DNA and the B–Z transition and may provide insight into the nature of Z-DNA interactions with small molecules such as specific ions or proteins essential to gene activity, thus leading to a better understanding of the functional role of Z-DNA in vivo.

Materials and Methods

Calf Thymus DNA Preparation. Calf thymus (CT) DNA was purchased from Sigma Chemicals, Inc., for control studies. This DNA is 56% AT and 44% GC and will maintain a right-handed conformation under high salt conditions (4 M NaCl). Solutions of CT DNA were prepared using the method of Chaires et al. (1982). The concentration of DNA solutions in terms of base pairs was determined by absorbance spectroscopy using the molar absorptivity, ϵ_{260nm}, of 13 200 M^{-1} cm^{-1}.

Synthetic DNA Preparation. Poly(dGdC)·poly(dGdC) (Lot No. QF817910) and poly(dGm^{5}dC)·poly(dGm^{5}dC) (Lot No. QH81793801) were 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. The methylated copolymer was reported to have a sedimentation coefficient, $S_{20,w}$, equal to 21.3, corresponding to an average length of 11 886 base pairs. Solutions were made by dissolving the solid sodium salts of these polymers [100 optical density units (OD) for poly(dGdC)·poly(dGdC) or 25 OD units for poly(dGm^{5}dC)·poly(dGm^{5}dC)] in 1 mL of 0.01 M NaCl/sodium phosphate buffer. Experimental solutions were prepared by adding a known amount of stock solution to phosphate buffer. Concentrations of polynucleotide solutions were determined by UV spectroscopy using the molar absorptivity value of 16 800 M^{-1} cm^{-1} at 254 nm for both polymers (Chaires, 1985).

Photoaffinity Probe. Ethidium monoazide was synthesized by the method of Graves et al. (1977) under photographic safelight conditions due to the photoreactive nature of the products. Approximately 0.1 mg of solid ethidium monoazide was dissolved in 0.5 mL of 0.01 M sodium phosphate, pH 7.0/0.001 M disodium EDTA/0.01 M NaCl. After thorough mixing, this solution was filtered through a 0.45-μm filter. A total of 100 μL of stock solution was diluted into 3 mL of buffer for experimental solutions. The concentration was determined spectrophotometrically using a molar extinction coefficient of 5220 M^{-1} cm^{-1} at 460 nm (Graves et al., 1977). All drug solutions were prepared fresh immediately prior to the experiment.

Covalent Attachment of Ethidium Monoazide. Samples of varying ratios of ethidium covalently attached to poly(dGdC)·poly(dGdC) or poly(dGm^{5}dC)·poly(dGm^{5}dC) were prepared in 0.01 M sodium phosphate, pH 7.0/0.001 M disodium EDTA/0.01 M NaCl under photographic safelight conditions. After equilibration for 60 min at 5 °C, the drug was covalently attached to the DNA via photolysis of the drug–DNA complex. The samples were photolyzed using two light boxes (Haake-Buchler Instruments) equipped with General Electric daylight No. F15T8-D lightbulbs delivering energy at a rate of ~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 by photolysis was 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 layered on the top of the columns and eluted by centrifuging for 1 h at 1000 rpm. Temperature was maintained at 5 °C throughout the purification process.

The amount of ethidium monoazide and DNA in the covalent complex was quantitated by UV–visible absorbance. The absorbance of each sample was measured at 500 and 260 nm in self-masking semi-micro UV cells with a 1-cm path length. The concentration of covalently attached drug was

calculated from the absorbance at 500 nm using a molar extinction coefficient of 4100 M^{-1} cm^{-1} (Graves et al., 1981). The fraction of the absorbance at 260 nm due to the drug was corrected to obtain accurate DNA concentrations. The fraction of DNA with ethidium monoazide covalently attached is expressed as r (defined as the ratio of drug covalently attached per base pair of DNA).

Equilibrium Studies of the Salt-Induced B to Z Transition. The effects of covalent attachment of ethidium monoazide on the B to Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) were examined. The transition was induced by the stepwise addition of solid sodium chloride to the prepared adducts. The DNA concentration of all samples was adjusted to 40 μM in 0.01 M NaCl/sodium phosphate buffer. The sodium chloride concentration was increased in 0.5 M increments up to a final concentration of 5 M for the B to Z transition of poly(dGdC)·poly(dGdC) and increments of 0.1 M up to a final concentration 1.5 M for poly(dGm^{5}dC)·poly(dGm^{5}dC). After the addition of salt, samples were heated to 37 °C in a water bath for 30 min to facilitate the B to Z conversion and then allowed to cool to room temperature before spectral acquisition.

The salt induced B–Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) is accompanied by a near inversion of the circular dichroism (CD) spectrum (Pohl & Jovin, 1972). Under low salt conditions, the CD spectrum is characterized by a positive band at 280 nm and a negative band at 253 nm. Upon transition to a left-handed conformation, the positive band is converted to a more intense negative band with a minimum at 290 nm. Similarly, the negative band at 253 nm is inverted to a positive band at 265 nm (Pohl & Jovin, 1972). In the presence of ethidium, the spectrum exhibits isoelliptic points at 250 and 295 nm (Walker et al., 1985). These wavelengths provide a convenient means of monitoring the B to Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) because of the large changes in ellipticity at 250 and 295 nm exhibited when the polynucleotides undergo a left- to right-handed transition.

Circular dichroism spectra were recorded on a JASCO J-500A spectropolarimeter utilizing an IF-500 A–D converter interfaced to an IBM PS-II computer. Spectra of 1-mL samples were recorded at 22 °C in a cylindrical cell with a 1-cm path length from 220 to 350 nm. A baseline was recorded for 0.01 M NaCl/sodium phosphate buffer and automatically subtracted from the subsequent spectra. The molar ellipticity, $[\theta]$, was calculated from

$$[\theta] = \frac{100\theta}{cl}$$

where θ is the ellipticity in degrees, c is the molar base pair concentration of polynucleotide, and l is the path length in centimeters.

The UV absorption spectrum of poly(dGdC)·poly(dGdC) also undergoes distinct changes upon conversion to a left-handed conformation. At high salt concentrations, the spectrum shows a decrease in the absorbance at 260 and an increase at 295 nm compared to the low salt spectrum. The 260 nm/295 nm absorbance ratio can be used to monitor the B to Z transition. This ratio ranges from 8.5 for B-DNA to 3.2 for Z-DNA (Pohl & Jovin, 1972; Chaires, 1985). Ultraviolet absorption data was recorded on a Varian Cary 2290 UV–visible spectrophotometer at 22 °C. Since ethidium also absorbs in the UV region of the spectrum, the ratio of $R°/R$ was used to monitor the transition of those samples containing drug, where R is the 260 nm/295 nm absorbance ratio and $R°$ is the A_{260}/A_{295} ratio at 0.01 M NaCl.

Results

Covalent Attachment of Ethidium to DNA. Prior to photolysis, ethidium monoazide forms a reversible complex with DNA identical to that of the parent ethidium bromide. However, upon photolysis, a fraction of the drug molecules complexed with DNA are covalently attached via generation of a reactive nitrene intermediate. The photolytic efficiency of this covalent attachment is expressed as the fraction of drug covalently bound per base pair of DNA following irradiation divided by the total amount of drug which was present (per base pair). Modification of the DNA appears linear until saturating levels of drug are reached. The slopes of the linear portion of the binding curves are used to calculate the photolytic efficiencies for covalent attachment of ethidium to poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC). The binding efficiency for poly(dGdC)·poly(dGdC) is 43%, which is in close agreement with the value reported by Graves et al. (1981) for covalent modification of calf thymus DNA by ethidium monoazide. The efficiency for poly(dGm^{5}dC)·poly(dGm^{5}dC) was demonstrated to be 67%, almost twice that of the unmethylated copolymer.

The differences in the efficiency of covalent attachment between poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) can be explained in part by differences in binding affinities. Walker et al. (1985) reported an apparent binding constant of 8.3×10^5 M^{-1} for the binding of ethidium bromide to poly(dGdC)·poly(dGdC) and 1.6×10^6 M^{-1} for poly(dGm^{5}dC)·poly(dGm^{5}dC). Assuming that differences in binding affinities of the parent ethidium are reflected in the binding affinities of the monoazide, ethidium monoazide also demonstrates a higher binding affinity for poly(dGm^{5}dC)·poly(dGm^{5}dC) (data not shown) indicating that more drug would be bound to methylated polymer at equilibrium than to poly(dGdC)·poly(dGdC). Considering that the efficiency of photolytic attachment is directly dependent on the amount of drug bound at equilibrium, a higher photolytic efficiency would be expected for ethidium with poly(dGm^{5}dC)·poly(dGm^{5}dC).

Inhibition of the B to Z Transition. At low salt (0.1 M NaCl), both the poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) are presumed to be in the B conformation as indicated by their characteristic CD spectra, and the fraction of DNA in the Z conformation is defined as 0%. At the highest salt concentration [(5 M NaCl for poly(dGdC)·poly(dGdC) and 1.5 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC)] the fraction of DNA in the Z conformation is defined as 100%, with both polymers entirely in the Z conformation.

Transition curves for the salt-induced B to Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) are shown in parts A and B, respectively, of Figure 2. These equilibrium studies demonstrate that covalent attachment of ethidium monoazide inhibits the extent of the B to Z transition of both poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC). In the absence of ethidium, a midpoint in the transition curve is approximately 2.5 M NaCl for poly(dGdC)·poly(dGdC) and 0.8 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC). Both of these values are in good agreement with previous studies (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981). Upon covalent modification with ethidium (azide) of the alternating copolymers, both the extent of reaction and the midpoint of the transition are demonstrated to be dependent on the amount of drug covalently attached to the DNA.

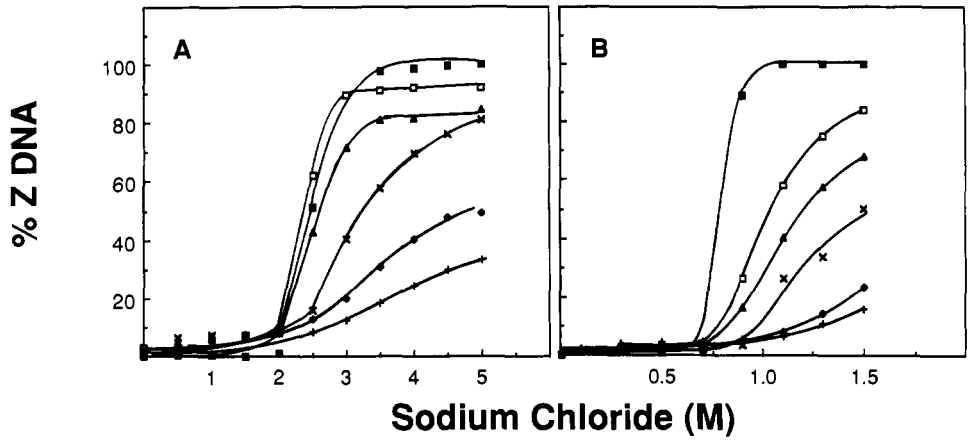

FIGURE 2: Transition curves for the salt-induced B to Z transition of (A) poly(dGdC)·poly(dGdC) and (B) poly(dGm^{5}dC)·poly(dGm^{5}dC). In panel A, the poly(dGdC)·poly(dGdC) is modified with the following ratios of covalently bound drug per base pair: ■, r = 0; □, r = 0.037; ▲, r = 0.070; ×, r = 0.110; ♦, r = 0.226; +, r = 0.293. In panel B, the poly(dGm^{5}dC)·poly(dGm^{5}dC) is modified by ethidium monoazide with ratios of covalently bound drug per base pair as follows: ■, r = 0; □, r = 0.042; ▲, r = 0.059; ×, r = 0.100; ♦, r = 0.152; +, r = 0.195).

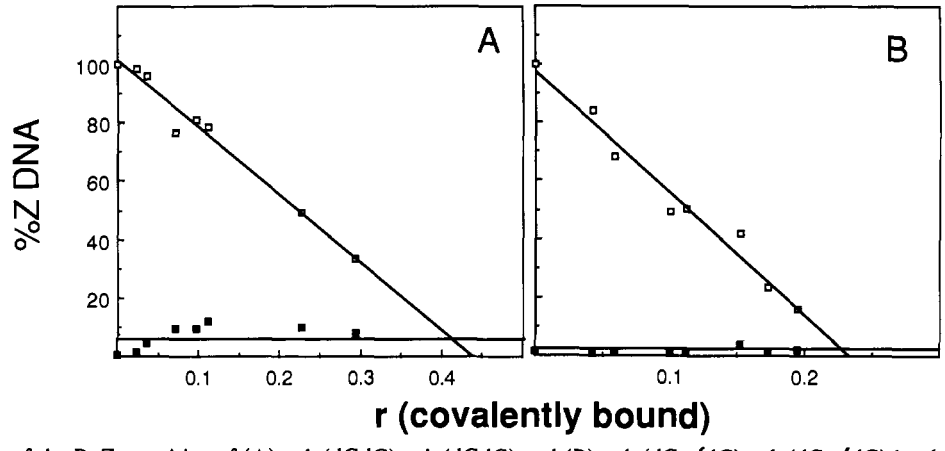

FIGURE 3: Inhibition of the B–Z transition of (A) poly(dGdC)·poly(dGdC) and (B) poly(dGm^{5}dC)·poly(dGm^{5}dC) by the covalent attachment of ethidium monoazide. The open squares (□) represent the amount of DNA in the Z conformation as a function of r (covalently bound drug) under Z-form conditions [5 M NaCl for poly(dGdC)·poly(dGdC) and 1.5 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC)]; the closed squares (■) represent the percent Z under low salt conditions (0.01 M NaCl).

A plot of the fraction of DNA in the Z conformation versus r (covalently bound ethidium per base pair) for poly(dGdC)·poly(dGdC) is shown in Figure 3A. Increasing the amount of drug results in a linear decrease in the fraction of DNA in the Z conformation. The high salt data (5 M NaCl) is shown to converge with the control low salt data (0.1 M NaCl) at an r value of 0.42 corresponding to one covalently bound ethidium per 2.4 base pairs. Theoretically, the B to Z transition of poly(dGdC)·poly(dGdC) would be completely inhibited at this ratio because the helix would be near saturation by ethidium, implying that 2 or 3 base pairs of poly(dGdC)·poly(dGdC) are held in a right-handed conformation upon the covalent attachment of one ethidium. Similarly, analyses of the ethidium-modified poly(dGm^{5}dC)·poly(dGm^{5}dC) reveal convergence at an r value of 0.22 for the B and Z conformations as shown in Figure 3B. This value corresponds to approximately 5 base pairs of DNA being locked into a right-handed conformation for each ethidium covalently attached to the poly(dGm^{5}dC)·poly(dGm^{5}dC). Interestingly, these values for the irreversibly bound ethidium are in close agreement with the results observed for reversible binding ethidium bromide and daunomycin by Walker et al. (1985) and Chaires (1986), respectively.

Energetics of the B to Z Transition. The energetics of the B to Z structural transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) can be characterized from the transition curves shown by Figures 2 and 3. The equilibrium transition can be interpreted according to the two-state model for an intramolecular change between two states as shown by (Pohl & Jovin, 1972; Pohl, 1983)

$$\mathrm{B} + n[\mathrm{NaCl}] \overset{K_{eq}}{\rightleftharpoons} \mathrm{Z} \qquad (1)$$

where B and Z represent molecules in the B and Z conformations and n is the coefficient of the salt concentration required to induce the right- to left-handed structural conversion (Amaratunga et al., 1990). The apparent and true equilibrium constants for the reaction are given by the equation

$$K_{eq} = \frac{[\mathrm{Z}]}{[\mathrm{B}][\mathrm{NaCl}]^n} = \frac{K_{app}}{[\mathrm{NaCl}]^n} \qquad (2)$$

which leads to

$$K_{app} = K_{eq}[\mathrm{NaCl}]^n$$

From K_{app}, the ΔG_{app} (the apparent free energy for the B to Z transition) can be determined by

$$\Delta G_{app} = -RT \ln K_{app} = -RT \ln \frac{\theta}{1-\theta} \qquad (3)$$

where θ is equal to the fraction of DNA in the Z conformation. Equation 3 can be rewritten as

$$\Delta G_{app} = -RT \ln (K_{eq}[\mathrm{NaCl}]^n) = \Delta G^\circ - nRT \ln [\mathrm{NaCl}] \qquad (4)$$

Table I: Free Energy Parameters for the Salt-Induced B to Z Transition of Poly(dGdC)·Poly(dGdC) and Poly(dGm^{5}C)·Poly(dGm^{5}C) as a Function of Covalently Attached Ethidium

r (bound)	$\Delta G°$ (kcal/mol)	ΔG_{app} (kcal/mol)[a]	ΔG_{app} (kcal/mol)[b]	n[c]
B to Z Transition of Poly(dGdC)·Poly(dGdC)				
0.000	6.7 ± 0.3	40.2	−3.4	12.3
0.023	5.7 ± 0.7	34.8	−3.1	10.7
0.037	4.6 ± 0.2	28.9	−2.7	8.9
0.070	3.2 ± 0.1	18.4	−1.4	5.6
0.096	3.0 ± 0.1	14.4	−0.8	4.1
0.110	2.5 ± 0.2	13.7	−0.4	4.1
0.226	2.2 ± 0.1	7.8	0.5	2.1
0.293	2.0 ± 0.1	5.7	0.9	1.3
B to Z Transition of Poly(dGm^{5}dC)·Poly(dGm^{5}dC)				
0.000	−1.3 ± 0.02	29.2	−4.06	>10.0
0.042	0.2 ± 0.10	15.8	−1.18	5.7
0.059	0.5 ± 0.05	13.9	−0.64	4.7
0.100	0.8 ± 0.03	11.4	−0.11	3.9
0.112	0.9 ± 0.02	11.1	−0.06	3.8
0.152	0.9 ± 0.10	7.9	0.35	2.5
0.173	1.1 ± 0.06	7.1	0.52	2.2
0.195	1.1 ± 0.12	5.4	0.70	1.6

[a] Evaluated graphically (plot of ΔG_{app} versus ln [Na$^+$]; determined n and $\Delta G°$ at [Na$^+$] = 0.01 M. [b] [Na$^+$] = 4 M NaCl. [c] n describes the differential counterion uptake between B and Z DNA conformations. [d] [Na$^+$] = 1.5 M NaCl.

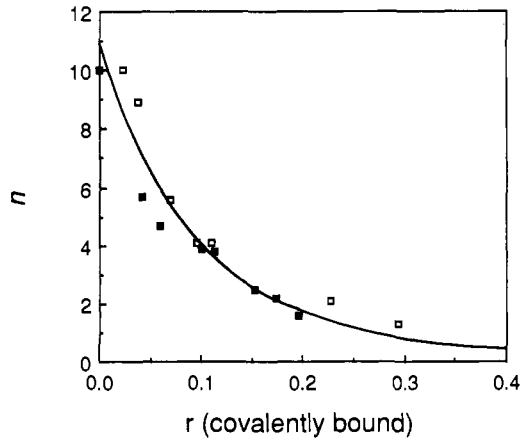

FIGURE 4: Graphical representation of n (coefficient of salt concentration required to induce right- to left-handed structural conversion) from eq 4 as a function of covalent modification of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) with ethidium. At r = 0, no drug present, n for the B to Z transition is approximately 12. With increasing amounts of covalently bound drug, n decreases to 1.

Plots of the B–Z transition free energy, ΔG_{app} (determined from eq 4 as a function of ln [NaCl]) were linear with a slope equal to $-nRT$ and an intercept $\Delta G°$ (figure not shown).

Table I lists the graphically determined values for n, $\Delta G°$, and ΔG_{app} for the B to Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) as a function of covalently attached ethidium. As the amount of covalently attached ethidium is increased, the value of n determined by the plot is demonstrated to decrease, as shown in Figure 4. In this case, n is defined as the coefficient of salt concentration required to induce the right- to left-handed structural conversion.

Free Energy of the B–Z Transition. The values of ΔG_{app} listed in Table I were calculated from the graphically determined values of $\Delta G°$ and n at the designated salt concentration. The $\Delta G°$ obtained from the y-intercept of a graph of ΔG_{app} versus ln [NaCl] is evaluated assuming a standard state concentration of 1 M NaCl, since $\Delta G_{app} = \Delta G°$ when ln [NaCl] equals 0. At 1 M salt concentration, the CD spectrum

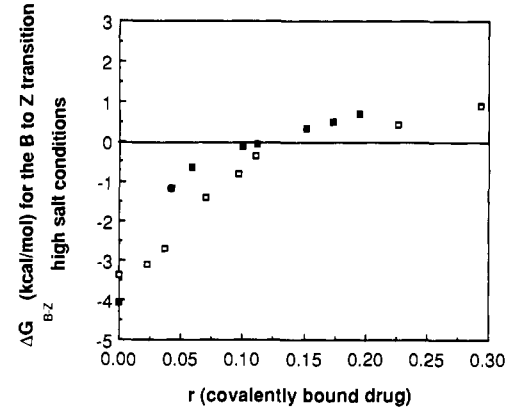

FIGURE 5: The free energy of the salt-induced B to Z transition plotted as a function of covalent modification of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC). All data were determined at 5 M NaCl for poly(dGdC)·poly(dGdC) and 1.5 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC), respectively. Increasing the levels of modification by ethidium results in a positive increase in the free energy of the B–Z transition, making the transition less favorable. Both alternating copolymers experience a change from negative to positive ΔG_{app} at a level of 1 drug bound per 10 base pairs.

of poly(dGdC)·poly(dGdC) reveals this copolymer to be in the B form; however, at 1 M NaCl, the poly(dGm^{5}dC)·poly(dGm^{5}dC) is well into conversion to the left-handed conformation. Thus, a $\Delta G°$ determined at 1 M NaCl does not provide a good measure of the relative stabilities of the B and Z conformations when poly(dGdC)·poly(dGdC) is compared with the methylated copolymer. To compensate for the structural transition at 1 M NaCl by the methylated copolymer, the ΔG_{app} value was calculated at 0.01 M NaCl, conducive to the B conformation for both alternating polynucleotides. The high salt concentrations were established as 4 M NaCl for poly(dGdC)·poly(dGdC) and at 1.5 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC). As shown in Table I, the free energy values for the B to Z transition (ΔG_{app}) calculated at 0.01 M NaCl are highly positive (>20 kcal/mol) for both poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) as expected because of the instability (or lack) of the Z conformation at low salt conditions. At 0.01 M NaCl, both copolymers should be in a right-handed conformation. As the amount of covalently bound ethidium is increased, the value of ΔG_{app} is shown to decrease, due most likely to structural perturbations of the DNA resulting from increasing levels of covalently attached drug (i.e., as it approaches an intercalated B conformation) rather than an intrinsic stabilization of the Z conformation.

At salt conditions which are conducive for the Z conformation to exist, the ΔG_{app} is negative (approximately −4 kcal/mol) for both poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) as shown in Figure 5, indicative of spontaneous adoption of the Z conformation. Covalent attachment of ethidium results in an increase in the free energy for the B to Z transition, demonstrating the effectiveness of the drug in inhibiting the B to Z transition and making the right-handed conformation more stable. As observed in Figure 5, the free energy associated with the B to Z transition for both alternating copolymers becomes positive at approximately 1 covalently bound drug per 10 base pairs.

Discussion

Photoaffinity analogues of ethidium have been previously used to study high-affinity binding sites in plasmid DNA (Coffman et al., 1982; Hardwick et al., 1984) and mutagenesis in mitochondrial DNA (Hixon et al., 1975; Fukunaga &

Yielding, 1979). The study presented here utilizes ethidium monoazide as a photoaffinity probe to examine the structure and energetics of the B–Z transitions of the synthetic polynucleotides poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC). Intercalating drugs have been widely used to probe the secondary and tertiary structure of DNA through structural and conformational perturbations arising upon complex formation. However, results from previous studies using reversible binding drugs are limited in scope due to their free dissociation from the DNA. Therefore, the observed effects on DNA structure upon drug binding provide an overall average of the individual effect of drug interactions. In addition, the changes in salt concentrations that are used to induce the left-handed conformation also drastically alter the binding affinity of cationic drugs. These limitations may be overcome through the use of photoaffinity analogues. Covalent attachment of ethidium effectively locks the drug–DNA complex and provides a novel method for studying the effects of drug binding on DNA structure, particularly the B–Z transition, in much greater detail.

These studies demonstrate that covalent attachment of ethidium monoazide to both poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) inhibits the salt-induced B to Z conformational transition. Intercalation of ethidium into the DNA duplex results in the stabilization of the right-handed DNA conformation. Covalent attachment of the drug within the intercalation site prevents this site from assuming the left-handed conformation. Figure 3 suggests that the covalent modification of poly(dGdC)·poly(dGdC) by ethidium monoazide effectively locks 2–3 base pairs of poly(dGdC)·poly(dGdC) into a right-handed conformation. Similarly, 4–5 base pairs of poly(dGm^{5}dC)·poly(dGm^{5}dC) are rendered irreversibly locked into a right-handed conformation around the ethidium–DNA intercalation site.

The B to Z transition curves shown in Figure 2 demonstrate a loss in cooperative nature of this transition as a function of the covalently bound ethidium. This decrease in the cooperativity of this transition can be interpreted in terms of the chain-length dependence of the transition. Pohl and Jovin demonstrated that the transition curves for oligomers of different chain length become steeper with increasing molecular weight. If the mechanism of the transition is explained in terms of a rate-limiting nucleation of a left-handed region followed by rapid propagation down the helix, a chain-length dependence is understandable. In the case of covalent modification of the alternating copolymers poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC), the covalent attachment of ethidium acts to effectively shorten the cooperative unit.

In addition, covalent attachment of ethidium results in significant effects on the free energy changes for the transition. Values shown as ΔG_{app} listed in Table I were calculated for poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC) at both B- and Z-form conditions. The large positive value of ΔG_{app} ($\sim$20 cal/mol) calculated at 0.01 M NaCl for the polymers confirms that the Z conformation is energetically unfavorable at low salt concentrations. The ΔG_{app} observed for poly(dGdC)·poly(dGdC) is larger than that of poly(dGm^{5}dC)·poly(dGm^{5}dC), demonstrating that methylation of cytosine results in a decrease in the stability between the B and Z forms as compared to the unmethylated polymer, which is consistent with the lower salt concentrations required for induction of the B to Z transition in the poly(dGm^{5}dC)·poly(dGm^{5}dC). The free energy of the transition (ΔG_{app}) is shown to increase as a function of increasing amounts of covalently bound drug and may be explained in terms of the formation of an "intercalated" B conformation (i.e., a B-form nucleation site). Upon saturation of the DNA helix with drug molecules, the intercalated B* conformation demonstrates slightly higher energy (ΔG_{app}) than the nonmodified B conformation. Hence, the effect of drug binding on the ΔG_{app} at 0.01 M NaCl is to increase the energy of the right-handed conformation rather than stabilization of the Z conformation. The influence of covalent attachment of ethidium on the ΔG_{app} at high salt concentrations is drastically different. In the absence of ethidium, a ΔG_{app} of ~ -4 kcal/mol for both polynucleotides indicates that the Z conformation is highly preferred. However, the ΔG_{app} values are shown to become more positive with increasing amounts of covalently attached ethidium. In fact, at 1 drug bound per 10 base pairs, this free energy of transition becomes positive [even in the presence of 5 M NaCl for poly(dGdC)·poly(dGdC) and 1.5 M NaCl for poly(dGm^{5}dC)·poly(dGm^{5}dC)] demonstrating that covalent attachment of ethidium results in stabilization of the right-handed conformation relative to the Z DNA conformation. Ethidium provides permanent nucleation sites for the intercalated B form even under conditions that highly favor formation of the Z conformation.

These results demonstrate that covalent attachment of ethidium monoazide is effective in inhibiting the B to Z transition of poly(dGdC)·poly(dGdC) and poly(dGm^{5}dC)·poly(dGm^{5}dC). Intercalation of ethidium and subsequent covalent attachment in situ results in an irreversible right-handed nucleation site, which effectively blocks the transition of the B to Z conformations. Propagation of the transition is interrupted by the right-handed binding sites resulting in a marked decreased cooperativity of the transition and a change in the effective Na^+ ion binding differential between the B and Z conformations of DNA.

Registry No. Poly(dGdC), 36786-90-0; poly(dGm^{5}dC), 51853-63-5; ethidium monoazide, 63783-82-4.

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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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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-

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