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Conversions of the left-handed form and the protonated form of DNA back to the bound right-handed form by sanguinarine and ethidium: A comparative study

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Abstract

The interaction of sanguinarine and ethidium with right-handed (B-form), left-handed (Z-form) and left-handed protonated (designated as H^L-form) structures of poly(dG–dC)·poly(dG–dC) and poly(dG–me^5dC)·poly(dG–me^5dC) was investigated by measuring the circular dichroism and UV absorption spectral analysis. Both sanguinarine and ethidium bind strongly to the B-form DNA and convert the Z-form and the H^L-form back to the bound right-handed form. Circular dichroic data also show that the conformation at the binding site is right-handed, even though adjacent regions of the polymer have a left-handed conformation either in Z-form or in H^L-form. Both the rate and extent of B-form to Z-form transition were decreased by sanguinarine and ethidium under ionic conditions that otherwise favour the left-handed conformation of the polynucleotides. The rate of decrease is faster in the case of ethidium as compared to that of sanguinarine. Scatchard analysis of the spectrophotometric data shows that sanguinarine binds strongly to both the polynucleotides in a non-cooperative manner under B-form conditions, in sharp contrast to the highly-cooperative binding under Z-form and H^L-form conditions. Correlation of binding isotherms with circular dichroism data indicates that the cooperative binding of sanguinarine under the Z-form and the H^L-form conditions is associated with a sequential conversion of the polymer from a left-handed to a bound right-handed conformation. Determination of bound alkaloid concentration by spectroscopic titration technique and the measurement of circular dichroic spectra have enabled us to calculate the number of base pairs of Z-form and H^L-form that adopt a right-handed conformation for each bound alkaloid. Analysis reveals that 2–3 base pairs (bp) of Z-form of poly(dG–dC)·poly(dG–dC) and poly(dG–me^5dC)·poly(dG–me^5dC) switch to the right-handed form for each bound sanguinarine, while approximately same number of base pairs switch to the bound right-handed form in complexes with H^L-form of these polynucleotides. Comparative binding analysis shows that ethidium also converts approximately 2 bp of Z-form or H^L-form to bound right-handed form under same experimental conditions. Since

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sanguinarine binds preferentially to alternating GC sequences, which are capable of undergoing the B to Z or B to H^L transition, these effects may be an important part in understanding its extensive biological activities.

Keywords: Sanguinarine; Ethidium; B to Z transition; B to H^L transition; Ligand–polymorphic DNA interaction

1. Introduction

DNA is a structurally polymorphic macromolecule that can adopt a variety of conformations, which show significant deviation from canonical right-handed DNA (B-DNA) depending on nucleotide sequence and environmental conditions [1]. Of the polymorphic structures so far known, perhaps the most striking example is the Z-DNA, which is a left-handed duplex conformation with Watson–Crick base pairing, easily inducible in alternating purine–pyrimidine sequences. The formation of Z-DNA was first observed by Pohl and Jovin [2] as an inversion of the circular dichroic bands of the B-form poly(dG–dC)·poly(dG–dC) in solutions of high NaCl concentration. Later, X-ray structure analysis of oligonucleotide crystals confirmed the left-handed Z-form [3]. Extensive studies have subsequently shown that Z-DNA can be generated in solutions of poly(dG–dC)·poly(dG–dC) and more easily in its methylated analogue under a variety of conditions, e.g. in the presence of divalent cations like Mg^{2+}, Ca^{2+} and Ni^{2+}, 60% ethanol, 40 μM concentration of hexamine cobalt chloride or millimolar concentrations of polyamines [4–6]. Both the chemistry and biology of Z-DNA are a matter of current interest — in part to understand the possible role of Z-DNA in gene expression [7]. Evidence for the existence of Z-DNA in vivo has been provided [8–13]. It has been described that right-handed B and left-handed Z conformations coexist in equilibrium in plasmids in *Escherichia coli* [8] and they have also been detected in chromosomes from *Drosophila* [9] and in metabolically active mammalian cells [10]. Moreover, sequences that can adopt Z-conformation are found in the enhancer regions of the SV-40 minichromosome [11,12] and between two transcription units alternatively exposed during the development of *Drosophila hydei* [13]. Since alternating CG sequences are among those required to obtain a Z-conformation [2,3], we tentatively consider that preferential binding to such sequences could be part of the mechanism used to inhibit cell processes like replication and transcription. X-Ray diffraction studies on oligonucleotide crystals grown under a variety of Z-forming conditions indicated several variants of left-handed Z-form [3,14], and these structures were also observed in solutions using circular dichroism as a tool.

Protonation of DNA has been studied for several years [15–18], but has gained considerable significance from the observation by Jovin and colleagues [19] of the dramatic increase of anti Z-DNA immunofluorescence intensity of polytene chromosomes upon short exposure to low pH and the observation of Chen [20] that a left-handed Z-DNA structure could be generated on protonation of poly(dG–dC)·poly(dG–dC). Our laboratory has been studying the protonation of natural and synthetic polymers in order to understand their structural polymorphism [21–25]. From extensive absorption spectroscopic and circular dichroic studies we have advanced a model with the formation of Hoogsteen base pairs with a change of the handedness of the helix at low pH [25]. Our model of left-handed structure with Hoogsteen base pairing was confirmed by the FTIR studies of Tajmir Riahi and coworkers [26]. More recently Otto and coworkers [27] investigated in considerable detail the pH-dependent structure of poly(dG–dC)·poly(dG–dC) employing Raman spectroscopy, absorption spectroscopy and circular dichroism. This study also confirms the model involving the change of handedness accompanied by formation of Hoogsteen base pairs in protonated structures [25]. With a large range of accessible conformations, it is most likely that many of these structures will be found to

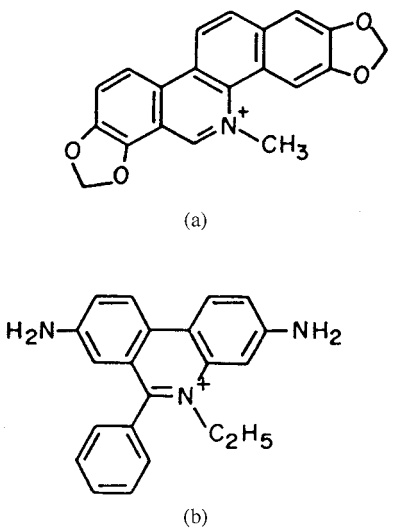

Fig. 1. Chemical structures of (a) sanguinarine and (b) ethidium.

have biological importance. Detailed studies on these structures will provide clues that facilitate discovery of their biological roles. The biological relevance of protonation of nucleic acid bases has also been acknowledged recently [28–33].

Sanguinarine (Fig. 1a), a benzophenanthridine alkaloid, is a planar molecule with a fused aromatic ring system and it is a widely distributed plant alkaloid with wide-ranging biological activities [34–41]. It has displayed excellent antimicrobial, antiplaque and antitumour activities [35–38]. It exhibits strong anti-inflammatory and antitubulin activities as well [39,40]. Its salt has been used in toothpastes and dental rinses [36,41]. Our laboratory has unequivocally demonstrated that sanguinarine exhibits pH-dependent structural equilibrium between the iminium (charged) and alkanolamine (uncharged) form with pK-value of 7.4 as revealed by spectrophotometric and spectrofluorimetric measurements [42,43]. The stability of these two forms was later confirmed by studies of Jones et al. [44]. We have also demonstrated that the sanguinarine iminium form binds to DNA by a mechanism of intercalation with a high preference to GC base pairs of B-form structures [45–51], while the sanguinarine alkanolamine form does not bind to DNA [52]. Our findings have been further confirmed by several other workers [53–55]. The focus of the present study is the interaction of sanguinarine (Fig. 1a) with the three polymorphic forms, namely the right-handed B-form, the left-handed Watson–Crick base paired (Z-form) and the left-handed Hoogsteen base paired protonated form (hereafter H^L-form) of poly(dG–dC) · poly(dG–dC) and poly(dG–me^{5}dC) · poly(dG–me^{5}dC). We have also studied the interaction of the known intercalator ethidium (Fig. 1b) with these three polymorphic structures for comparison under identical conditions, as ethidium is structurally close to sanguinarine and its binding to nucleic acids provides a general model for the biological activities of a number of intercalating agents.

2. Materials and methods

2.1. Materials

Sanguinarine chloride was obtained from Aldrich (Milwaukee, WI, USA). Ethidium bromide was a product of Sigma Chemicals Co. (St. Louis, MO, USA). These compounds were used without further purification as no detectable impurities were observed by TLC and [^{1}H]NMR. Solutions of sanguinarine and ethidium were freshly prepared each day and were always kept protected in the dark to prevent any light-induced changes. A molar extinction coefficient (ε) of 30 700 M^{-1} cm^{-1} at 327 nm in 0.1 N HCl for sanguinarine and 5680 M^{-1} cm^{-1} at 480 nm for ethidium was used for determining their concentrations. No deviation from Beer's law was seen in the concentration range employed in this study.

Poly(dG–dC) · poly(dG–dC) (hereafter poly[d(G–C)]) was a product of Sigma Chemicals Co., MO, USA, while poly(dG–me^{5}dC) · poly(dG–me^{5}dC) (hereafter poly[d(G–me^{5}C)]) was obtained from Pharmacia Biotech, Piscataway, NJ, USA. These polymers were used as such after checking their nativeness by ultraviolet (UV) and circular dichroic (CD) spectral measurements. Their concentrations in terms of nucleotide phosphate were estimated spectrophotometrically by using known molar extinction coefficients (ε) of 8400 M^{-1} cm^{-1} at 254 nm for poly[d(G–C)] and

7000 M^{-1} cm^{-1} at 255 nm for poly[d(G–me^{5}C)] as reported [25,56,57].

Citrate–phosphate (CP) buffer containing 5 mM Na_2HPO_4 was adjusted to the specified pH using citric acid [25,51]. This buffer provided constant [Na^+] of 10 mM. CP buffer of pH 5.2 was used for studies with the B-form, and formation of Z-DNA structure and its interaction with sanguinarine, as sanguinarine persists fully as the charged iminium form at this pH [42,52]. Formation of H^L-form structures and its interaction studies were performed at 10°C in CP buffer of pH 3.4. Analytical grade reagents and glass-distilled de-ionized water were used throughout. The buffer solutions were always passed through either 0.22-μM or 0.45-μM Millipore filters to remove any particulate matter.

2.2. Methods

2.2.1. Absorbance spectral measurement

All UV-Vis absorbance studies were made on a Shimadzu model UV-260 automatic recording double-beam spectrophotometer (Shimadzu Corporation, Japan) in a thermoelectrically controlled cell holder using a 1-cm path-length in matched quartz cuvettes. A thermoprogrammer (KPC 5) in conjunction with a temperature controller (SPR 5) was used to maintain the sample temperature.

2.2.2. Circular dichroic measurements

CD spectra were recorded on a Jasco J-720 spectropolarimeter equipped with a thermal programmer (PTC-343) and controlled by a Compaq PC 486. Rectangular quartz cells of a 1-cm path-length were used. The CD unit was routinely calibrated using solutions of d-10 ammonium camphor sulphonate. The CD spectra obtained are generally the average of four repetitive scans and the molar ellipticity [θ] is reported as degrees cm^2 $dmol^{-1}$ [25,56,57] in terms of polynucleotide concentration. CD titrations were performed concurrently with optical titration.

2.2.3. Formation of left-handed (Z) DNA structure

The B to Z transition was measured by absorption and CD spectroscopy. Before initiating interaction studies, the B to Z transition potentials of the poly[d(G–C)] and poly[d(G–me^{5}C)] samples were confirmed using individual samples under the three known conditions, namely, NaCl, $MgCl_2$ and $[Co(NH_3)_6]Cl_3$ with continuous stirring of the solution [57]. An equilibrium time of 40 min was generally allowed after initiating the conformational transition. UV and CD spectra were recorded before and after B to Z transition. The ratio of absorbance at 260 nm to that at 295 nm was used as a measure of the B to Z transition. Poly[d(G–C)] is known to aggregate under certain conditions in the presence of $[Co(NH_3)_6]Cl_3$ [4,5], but in our buffer conditions and the concentration range used here no such aggregation was noticed. Furthermore, our CD spectra does not indicate any light scattering at wavelengths (λ) of 350 nm in these samples, thus ruling out the question of aggregation under these conditions.

2.2.4. Formation of left-handed (H^L) DNA structure

The B to H^L transition was measured by absorption and CD as described earlier [25] by slowly adding the polymer stock solution to the 10 mM CP buffer, pH 3.4 maintained at 10°C under constant stirring. Dry nitrogen gas was purged through the sample chamber to avoid moisture condensation on the optical windows. The structural transition from B to H^L-form followed very fast kinetics and was observed to be completed within 1 s. The hypochromicity change at 255 nm during B to H^L transition was generally approximately 23–25%, resulting in a characteristic UV spectrum for the protonated poly[d(G–C)] and poly[d(G–me^{5}C)] duplexes [25]. The duplex nature of the H^L structure was further confirmed by thermal melting studies [25]. The T_m of the H^L forms of poly[d(G–C)] and [d(G–me^{5}C)] in CP buffer, pH 3.4, were 41°C and 54°C, respectively.

2.2.5. Spectrophotometric titrations

Titration experiments were performed using the Shimadzu spectrophotometer with the temperature controller (SPR 5) to maintain the sample temperature. The binding studies with the B- and Z-form polynucleotides were performed at 20°C, while that with the H^L-form was done at 10°C. Dry nitrogen gas was continuously purged

throughout the course of the titration at 10°C. Sufficient stock of polynucleotide solution was added to the buffer in a 1-cm path-length cuvette to yield a concentration of 40 μM. Successive aliquots from a stock solution of sanguinarine was added to the cuvette at 30-min intervals. The amount of free and bound sanguinarine was determined following the methods described by Chaires et al. [58]. Following each addition of the alkaloid, the absorbance at the characteristic peak, 327 nm, and the isosbestic point, 353 nm, were recorded after 30 min equilibrium.

The total alkaloid (C_T) present was calculated from

$$C_T = A_{353}/\varepsilon_{353} \quad (1)$$

where $\varepsilon_{353} = 14\,520\ M^{-1}\ cm^{-1}$ at the isosbestic point for sanguinarine.

The expected absorbance at 327 (A^o) is

$$A^o = C_T\,\varepsilon_{f327} \quad (2)$$

The difference in A^o and the observed absorbance was then used to calculate the amount of bound alkaloid as

$$C_B = A/\Delta\varepsilon = (A^o - A_{obs})/(\varepsilon_{f327} - \varepsilon_{B327}) \quad (3)$$

where $\varepsilon_{f327} = 30\,700\ M^{-1}\ cm^{-1}$ and $\varepsilon_{B327} = 15\,430\ M^{-1}\ cm^{-1}$ for sanguinarine.

ε_B was determined independently by adding a known quantity of sanguinarine to a large excess of DNA and assuming total binding.

The amount of free drug was then estimated by the difference,

$$C_f = C_T - C_B \quad (4)$$

Similarly for ethidium, the absorbance at its characteristic peak, 480 nm, and isosbestic point, 511 nm, was noted after each addition. C_T and C_B were calculated as described above using $\varepsilon_{f480} = 5680\ M^{-1}\ cm^{-1}$, $\varepsilon_{B480} = 2780\ M^{-1}\ cm^{-1}$ and $\varepsilon_{511} = 4010\ M^{-1}\ cm^{-1}$ for free, bound and isosbestic point, respectively.

2.2.6. Data analysis

The binding data were cast into the form of Scatchard plots of r vs. r/C_f, where r is the number of ligand molecules bound per mole of nucleotide and C_f is the molar concentration of the free ligand. r is calculated from $r = C_B/P$, where P is the DNA concentration in nucleotide phosphate. The binding isotherms with the B-form were fit to the neighbour exclusion model [59]

$$r/C_f = K'(1 - nr)[(1 - nr)/(1 - (n - 1)r)]^{(n-1)} \quad (5)$$

where K' is the binding constant to an isolated site and n is the neighbour exclusion parameter [59]. Fitting was performed using the programme SCATPLOT, Version 1.2 [60], which works on the algorithm described by Nandy et al. [61] that generates the best fit K' and n values corresponding to the minimum least squares variance between theoretical and experimental values.

2.2.7. Kinetics of the B to Z transition

The kinetics of the B to Z transition were measured by monitoring the change in the absorbance of poly[d(G–C)] and poly[d(G–me^{5}C)] solution at 295 nm at 20°C in the Shimadzu spectrophotometer. Poly[d(G–C)] and poly [d(G–me^{5}C)] at a concentration of 50 μM was maintained stirred at 20°C in the CP buffer (10 mM NaCl). The reaction was initiated by the addition of $[Co(NH_3)_6]Cl_3$ solution to give a final concentration of 40 μM for poly[d(G–C)] and 20 μM for poly[d(G–me^{5}C)], respectively. In order to examine the role of the alkaloid on the B to Z transition, the B-form of the polymers were added to a solution of $[Co(NH_3)_6]Cl_3$ containing the alkaloid, and the absorbance at 295 nm was recorded with constant stirring till no change in absorbance was observed. In another set of experiments, the transition was interrupted by the addition of sanguinarine or ethidium at different intervals. In all experiments, the same amount of the ligand was added to the reference solution also.

3. Results

3.1. CD and UV-spectral characteristics of B, Z and H^L-forms of poly[d(G–C)] and poly[d(G–me^{5}C)]

The CD spectra of the polynucleotides, poly[d(G–C)] and poly[d(G–me^{5}C)], in the B, Z and H^L-conformations are shown in Fig. 2. The CD spectrum of the B-form (curve 1) is characterized by a positive band in the 275-nm region followed by a large negative band around the 250-nm region. This CD spectrum undergoes a large change as the polymer switches from the right-handed B to either the left-handed Watson–Crick base paired Z-form (curve 2) or to the left-handed Hoogsteen base paired H^L-form (curve 3). In the case of B to Z transition, the positive band, observed in the 275-nm region of the B-form, is replaced by a more intense negative band in the 290–300-nm region and the negative 250-nm band is replaced by a positive band around 265 nm. Curve 2 in Fig. 2a,b represents the Z-form CD spectrum generated under conditions of 40 μM $[Co(NH_3)_6]Cl_3$ in the case of poly[d(G–C)] and 20 μM $[Co(NH_3)_6]Cl_3$ in the case of poly[d(G–me^{5}C)]. The actual shape and magnitude of the negative and positive bands of the Z-form are dependent on the condition of formation (cf. Section 2). Our Z-form CD spectra are in good agreement with that reported in the literature by previous workers [4,62,63]. In the case of B to H^L transition a negative band appears around 300 nm for poly[d(G–C)] (Fig. 2a) and around 310 nm for poly[d(G–me^{5}C)] (Fig. 2b) followed by a broad positive ellipticity in the 250–280-nm region and that is in conformity with our earlier observations [25]. The ellipticity of the negative band of the H^L-form is lower compared to the negative band of the Z-form. These three polymorphic forms exhibit a single sharp isoelliptic point at 277 and 281 nm in poly[d(G–C)] and poly[d(G–me^{5}C)], respectively, indicating a complete transition equilibrium of each structure from the B-form.

The UV spectra of these three polymorphic structures are depicted in the inset of Fig. 2a,b. The conversion of the B-form to Z- or H^L-form is characterized by significant changes in the nature

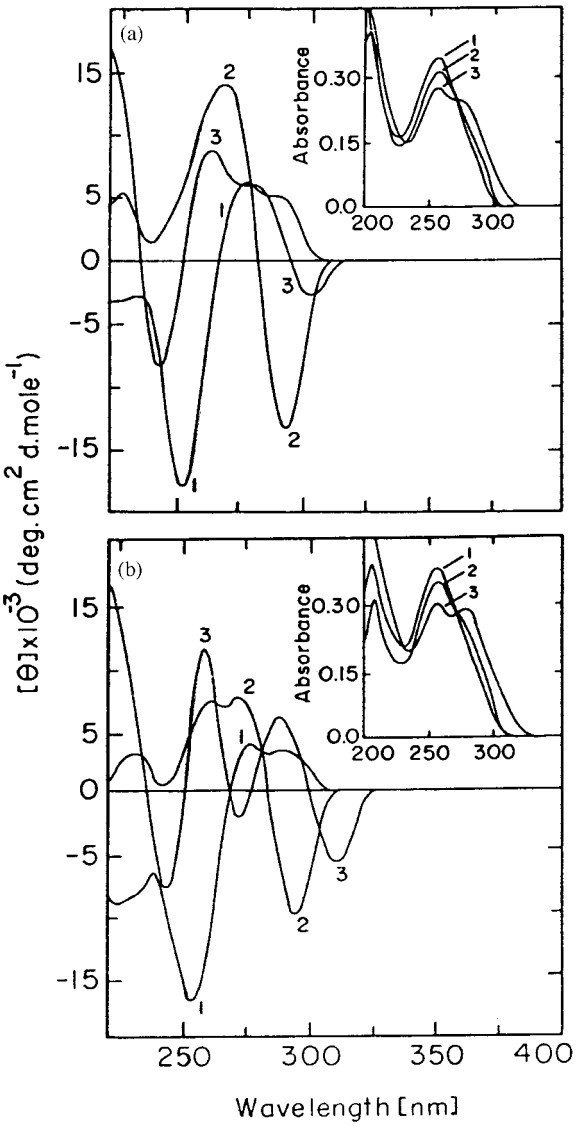

Fig. 2. CD spectra of (a) poly[d(G–C)] (40.33 μM) and (b) poly[d(G–me^{5}C)] (49.60 μM) in B-form (curve 1), in Z-form (curve 2) and in H^L-form (curve 3). Inset: UV spectra (a) poly[d(G–C)] (42.0 μM) and (b) poly[d(G–me^{5}C)] (55.0 μM) for B-form (curve 1), Z-form (curve 2) and H^L-form (curve 3).

and intensity of the UV spectrum in both the polymers particularly in the 295-nm region and is more pronounced in the H^L spectrum. Between the two polymers, the changes in the UV spectra were more pronounced in the methylated polymer. Like in the CD data, here also the three

polymorphic forms in both the polymers exhibit a single sharp isosbestic point at 271 nm, indicating the equilibrium of each of these duplex forms.

3.2. CD spectral changes of interaction of sanguinarine with the B, the Z and the H^L-form

3.2.1. B-Form–sanguinarine interaction

The CD spectral changes associated with the interaction of sanguinarine with poly[d(G–C)] and poly[d(G–me^5C)] are depicted in Fig. 3a and 3b, respectively. Binding of sanguinarine perturbs the B-form CD spectra of both the polymers. A large increase in the ellipticity of the long wavelength CD band (280 nm) was observed on binding. In the case of poly[d(G–C)], the ellipticity of this band increased by almost five times the initial value from $\sim 6300°$ cm^2 $dmol^{-1}$ to approximately

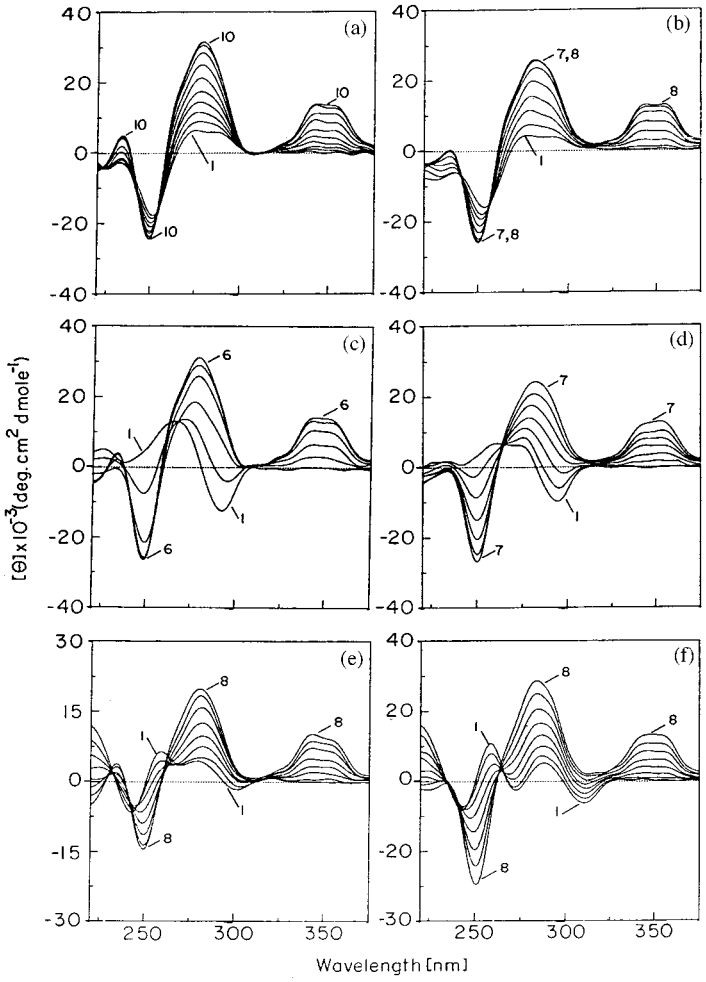

Fig. 3. Representative CD spectra resulting from the interaction of sanguinarine with B-form, Z-form and H^L-form of poly[d(G–C)] (40.33 μM) and poly[d(G–me^5C)] (49.60 μM): (a) curves 1–10 denote B-form poly[d(G–C)] treated with 0, 3.09, 4.63, 6.17, 7.71, 9.24, 10.77, 12.29, 13.82 and 15.34 μM of sanguinarine; (b) curves 1–8 denote B-form poly[d(G–me^5C)] treated with 0, 2.21, 4.41, 6.60, 8.79, 10.97, 13.13 and 15.29 μM of sanguinarine; (c) curves 1–6 denote Z-form poly[d(G–C)] treated with 0, 3.92, 7.83, 11.73, 15.60 and 19.46 μM of sanguinarine; (d) curves 1–7 denote Z-form poly[d(G–me^5C)] treated with 0, 2.20, 4.39, 6.58, 8.76, 10.94 and 15.28 μM of sanguinarine; (e) curves 1–8 denote H^L-form poly[d(G–C)] treated with 0, 2.39, 4.77, 7.15, 9.52, 11.89, 14.25 and 18.61 μM of sanguinarine; and (f) curves 1–8 denote H^L-form poly[d(G–me^5C)] treated with 0, 2.20, 4.39, 6.57, 8.35, 10.90, 13.05 and 15.19 μM of sanguinarine, respectively. The expressed molar ellipticity is based on polynucleotide concentration.

32 000° cm^2 $dmol^{-1}$ at saturation, which was achieved at a D/P (drug/DNA nucleotide phosphate molar ratio) ~0.34. In poly[d(G–me^5C)] also sanguinarine binding enhanced the long wavelength band ellipticity by approximately six times, from an initial 4300° cm^2 $dmol^{-1}$ to 26 700° cm^2 $dmol^{-1}$, showing a strong perturbation in the structure. Concomitant with the increase in rotational strength, this band became sharper also. The negative band intensity ~250 nm in both cases was relatively less perturbed and showed a decrease in ellipticity on sanguinarine binding. Comparatively, the changes in this band, however, were also more pronounced in the poly [d(G–me^5C)]. An isoelliptic point was observed at ~255 nm in both cases, indicating an equilibrium between the alkaloid-bound and the free polymer conformations. The 235–240-nm shoulder in the CD spectra also enhanced in ellipticity, although to a lesser extent in both the polymers.

3.2.2. Z-Form–sanguinarine interaction

The CD spectral changes associated with the interaction of sanguinarine with the Z-form of these polymers are depicted in Fig. 3c,d. On addition of the alkaloid, dramatic changes were observed in the 295-nm negative band which at first rapidly reduced in ellipticity, blue shifted, crossed over to the positive side and thereafter increased in ellipticity. At D/P ~0.19, the band maximum was centred around 279 nm which further enhanced in ellipticity and attained saturation at D/P ~0.49. At saturation, the features of this band in terms of ellipticity and wavelength maximum were similar to that seen with sanguinarine-bound B-form CD spectrum (Curve 10, Fig. 3a). The positive CD band (~267 nm) in the Z-form, on the other hand, partially reduced in ellipticity with concomitant blue shift to form a band around 250 nm similar to the negative 250-nm band of the B-form. At saturation, both intensity and wavelength maximum of this band were similar to that of the sanguinarine-bound B-form CD (Curve 10 of Fig. 3a). Two isoelliptic points were observed in the series of spectra in the case of the poly[d(G–C)], one at 268 nm and another at 255 nm while in the case of poly[d(G–me^5C)] a single isoelliptic point at 265 nm was seen.

3.2.3. H^L-Form–sanguinarine interaction

The H^L-form CD spectra of both the polymers were strongly perturbed on interaction with sanguinarine. The changes are depicted in Fig. 3e,f. The broad positive band in the 260–290-nm region enhanced in ellipticity and became a single sharp band with maximum around 280 nm. Concomitantly, the small negative band around 300 nm disappeared, and the negative band at 243 nm red shifted and enhanced in ellipticity. In the case of poly[d(G–C)], a saturation in the CD spectral changes was observed at D/P ~0.47 and the CD spectrum at this stage had features similar to a sanguinarine-bound B-form CD except that the ellipticity values of the peaks were slightly lowered. In contrast, the situation in the poly[d(G–me^5C)] was different; the final CD spectrum here, although resembling in shape its B-form-bound spectrum, had higher ellipticity values than that observed with the B-form.

3.3. Extrinsic CD spectra of sanguinarine–DNA interaction

Sanguinarine is an optically inactive molecule as it does not have any asymmetric centre. Consequently it does not have any CD spectrum in the entire UV-Vis range. But on binding to DNA, sanguinarine acquires optical activity manifested by the appearance of a positive CD band in the 320–370-nm region. In the case of B-form, the extrinsic CD of the fully-bound sanguinarine (Fig. 3a,b) had an ellipticity around 14 500 deg. cm^2 $dmole^{-1}$ with the wavelength maximum ~346 nm in both poly[d(G–C)] and poly[d(G–me^5C)]. With the Z-form (Fig. 3c,d) and the H^L-form (Fig. 3e,f) also at saturation, the extrinsic CD obtained for bound sanguinarine molecules had almost similar values as seen with the B-form. The shape and the nature of the extrinsic CD were also identical in all the three cases except for minor variations.

3.4. CD spectral studies on the interaction of ethidium with the B, the Z and the H^L-forms

In Fig. 4, the CD spectral changes observed on the interaction of ethidium with the B-, Z- and

H^L-forms of poly[d(G–C)] and poly[d(G–me^5C)] are depicted. With the B-form (Fig. 4a,b), CD spectral changes indicate increase in ellipticity of positive and negative bands suggesting intercalative binding of the ethidium. An extrinsic CD band is developed in the 300–360-nm region indicating the induction of CD in the bound ethidium molecules. In Fig. 4c,d, the CD changes with the Z-form of the polymers are depicted. The changes were grossly similar, representing the switching of Z-form conformation to the B-form conformation and the binding of ethidium to the B-form. In Fig. 4e,f, the interaction of ethidium with the H^L-form is depicted. The spectral changes here, however, were significantly different in the two polymers and also from that observed with the Z-form.

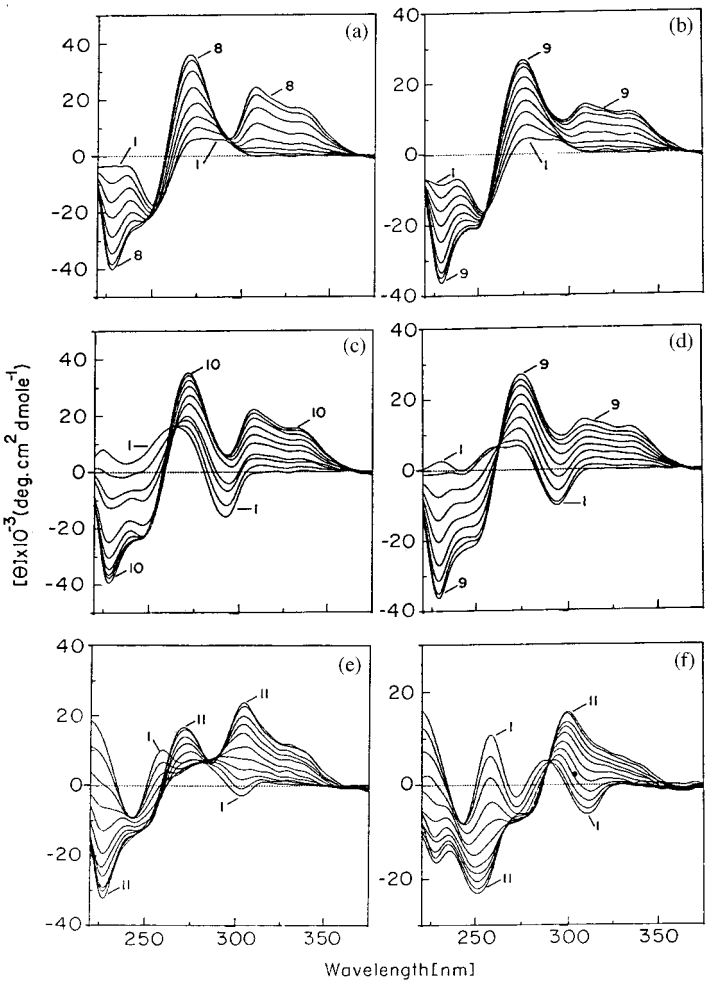

Fig. 4. Representative CD spectra resulting form the interaction of ethidium with B-form, Z-form and H^L-form of poly[d(G–C)] (40.84 μM) and poly[d(G–me^5C)] (49.60 μM): (a) curves 1–8 denote B-form poly[d(G–C)] treated with 0, 2.36, 4.72, 7.06, 9.40, 11.74, 14.06 and 16.38 μM of ethidium; (b) curves 1–9 denote B-form poly[d(G–me^5C)] treated with 0, 2.22, 4.43, 6.63, 8.83, 11.02, 13.20, 15.38 and 17.55 μM of ethidium; (c) curves 1–10 denote Z-form poly[d(G–C)] treated with 0, 3.54, 5.89, 8.24, 10.57, 12.90, 15.22, 17.53, 18.69 and 19.84 μM of ethidium; (d) curves 1–9 denote Z-form poly[d(G–me^5C)] treated with 0, 2.22, 4.43, 6.63, 8.03, 11.02, 13.20, 15.38 and 17.55 μM of ethidim; (e) curves 1–11 denote H^L-form poly[d(G–C)] treated with 0, 2.35, 4.70, 7.04, 9.36, 11.69, 13.99, 16.30, 18.58, 20.86 and 22.0 μM of ethidium; and (f) curves 1–11 denote H^L-form poly[d(G–me^5C)] treated with 0, 2.20, 4.41, 6.67, 8.90, 10.94, 12.97, 15.10, 17.21, 19.33 and 21.43 μM of ethidium, respectively. The expressed molar ellipticity is based on polynucleotide concentration.

With the GC polymer, the presence of ethidium produces changes in all the bands, but the extent of changes are less compared to that seen in B- and Z-forms. The spectrum obtained at the saturation D/P had much lower ellipticity values when compared with the corresponding spectra of B- and Z-form. Nevertheless, the extrinsic CD band had an ellipticity almost similar to that obtained in the B- and Z-form, but the maximum is seen at 304 nm here against 308 nm in B- and Z-forms. In the case of poly[d(G–me^{5}C)] the spectral changes were different from that seen with poly[d(G–C)]. The 259-nm band reduced in ellipticity and at saturation it became a negative band around 251 nm. No positive band characteristic of the B form was developed around 275 nm. The fully-bound spectrum of the polymer was clearly different from that seen in the H^L-form of the GC polymer and also different from that with the Z-form. Significantly, the extrinsic CD of the bound ethidium did not show any difference in ellipticity. The only noticeable difference was that the maximum was blue shifted by approximately 4 nm from maximum at 308 nm.

3.5. Switching of base pairs from a Z- or H^L-form to a right-handed helical form on binding of sanguinarine and ethidium

The variation of molar ellipticity [θ] at 295 nm in the case of Z to right-handed helical form and at 300 nm in the case of H^L to right-handed helical form transition was used to derive the number of base pair switching to the right-handed form for each molecule of bound sanguinarine or ethidium. In Fig. 5 the variation of molar ellipticity vs. r for the transition of Z to B and H^L to B-form is compared with the molar ellipticity change in the case of B-form binding. In Fig. 5a,b, the data on the transition of Z-form and the H^L-form of poly[d(G–C)] to the B-form on interaction with sanguinarine are compared with the B-form. With the Z-form the molar ellipticity exhibits a smooth change from a large negative value to positive values as a function of r and converge with B-form data at an $r \sim 0.20$. In the case of the H^L-form also, the values of the molar ellipticity at 300 nm meets the B-form data at an

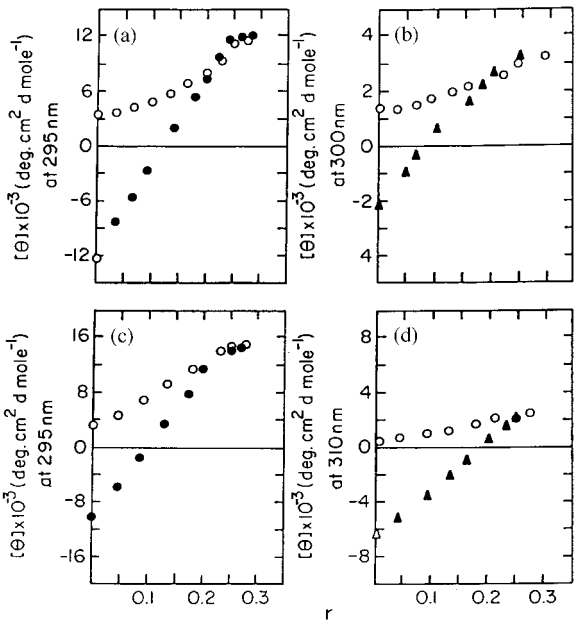

Fig. 5. Plot of molar ellipticity values at different wavelengths as a function of r for the interaction of sanguinarine with B-form (○), Z-form (●) and H^L-form (▲) of 40.33 μM poly[d(G–C)] in (a) and (b) and of 49.60 μM poly[d(G–me^{5}C)] in (c) and (d). Molar ellipticity was calculated from Fig. 3.

$r \sim 0.19$. From this the number of base pairs switching to the right-handed helical form for each bound sanguinarine can be deduced to be approximately 2–3 bp.

The data for the binding of sanguinarine to the Z- and H^L-form of poly[d(G–me^{5}C)] is plotted along with that of the B-form in Fig. 5c,d. Here the variation of molar ellipticity vs. r was measured at 295 nm and at 310 nm for Z-form and H^L-form, respectively. The Z-form molar ellipticity change meets the B-form data near $r \sim 0.20$ while the H^L-form data converge with the B-form at $r \sim 0.25$ indicating that approximately 3 and 2 bp of this polymer in the left-handed Z-form and H^L-form, respectively, are converted to the right-handed B-form for each bound sanguinarine. Under identical conditions, the data for poly[d(G–C)] interaction with ethidium (not shown) also indicate that the plots of variation of molar ellipticity with r in the case of Z- and H^L-form meets with the bound right-handed helical form at $r \sim 0.3$, suggesting the conversion of $\sim$2 bp to the right-handed helical form per bound ethidium.

In order to verify whether pH has any influence on the number of base pairs converted from the hexamine cobalt chloride induced Z-form to bound B-form by ethidium, we have performed CD experiments on the interaction of ethidium with Z-form poly[d(G–C)] under conditions of pH 8.0 in 40 μM $[Co(NH_3)_6]Cl_3$ and 50 mM Na^+ concentration as reported by Walker et al. [63]. It was observed from the plot of $[\theta]$ vs. r (not shown) that under these conditions ethidium binds cooperatively to the Z-form of poly[d(G–C)] and approximately 25 bp of the polymer are converted from the left-handed Z-form to the bound right-handed form per bound ethidium. However, under these conditions, sanguinarine interaction with the Z-form of poly[d(G–C)] could not be performed as sanguinarine exists in the alkanolamine form [42].

3.6. Kinetics of B to Z transition and the effect of sanguinarine

To support the conformational switching from left-handed Z to right-handed B structure observed in CD on binding of sanguinarine, we performed kinetic experiments in UV monitoring the change in absorbance at 295 nm, which is generally considered to be a diagnostic feature of the B to Z transition. The variation of absorbance with time is presented in Fig. 6. Fig. 6a shows the effect of different concentration of sanguinarine on the rate of the B to Z transition in poly[d(G–C)]. The reaction was initiated by adding poly[d(G–C)] solution to a buffered solution containing 40 μM of $[Co(NH_3)_6]Cl_3$, with or without appropriate amounts of sanguinarine. It can be seen that varying the amount of sanguinarine slows down the transition to different extents, while a complete inhibition of the transition was observed at $D/P = 0.3$ ($r = 0.23$). Fig. 6b depicts the reversal of the B to Z transition, interrupted by the addition of the same amount of the alkaloid at different time intervals, as indicated by the arrows. The data shows that adding the alkaloid at any stage could completely inhibit the B to Z transition. Similarly, the kinetics of the B to Z transition of poly[d(G–me^{5}C)] in the presence of sanguinarine was studied. The data is presented

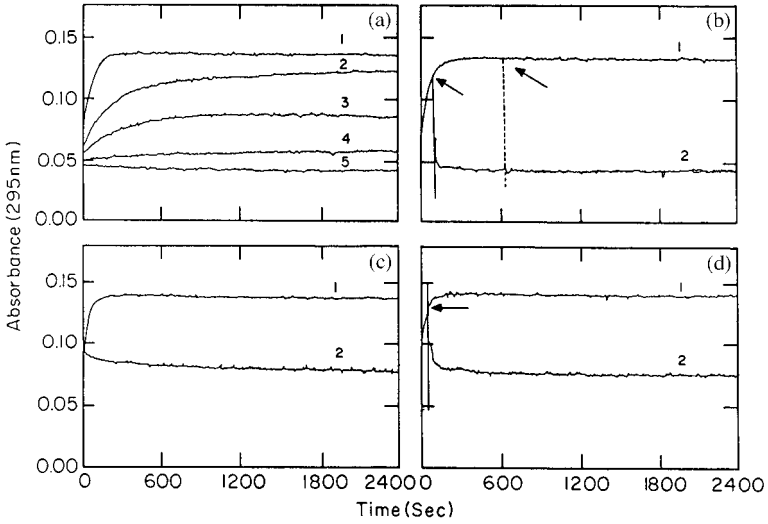

Fig. 6. Kinetic analysis of B to Z transition of 49.0 μM of poly[d(G–C)] and 51.0 μM poly[d(G–me^{5}C)] in absence and in presence of different amounts of sanguinarine. The reaction was initiated by adding polynucleotide to the buffer containing different amounts of sanguinarine as represented in (a) by curves 1 (0 μM), 2 (2.45 μM), 3 (4.90 μM), 4 (9.80 μM) and 5 (14.70 μM) and (b) by curves 1 (0 μM) and 2 (15.30 μM). Reversal of the B to Z transition by sanguinarine is presented in (b) and (d) for poly[d(G–C)] and poly[d(G–me^{5}C)], respectively. The transition was initiated as described above but was interrupted by adding sanguinarine at the points indicated by the arrows. (b) and (d) represent the change in absorbance in the absence (curve 1) and in the presence (curve 2) of 14.70 μM and 15.30 μM sanguinarine, respectively.

in Fig. 6c,d. Sanguinarine slows down the B to Z transition here also and a complete inhibition was observed at D/P = 0.3 ($r = 0.26$). Again, the B to Z transition was also interrupted on addition of alkaloid at different time intervals (Fig. 6d), as in the case of poly[d(G–C)].

The kinetics of the B to H^L structural transition followed a very fast kinetics which could not be monitored in the time scale of our spectrophotometer. However, in an indirect experiment, we prepared a complex of sanguinarine-B-form poly[d(G–C)] (saturation D/P) and this was then transferred into CP buffer of pH 3.4. We did not observe the formation of H^L structure in CD experiments indicating that the alkaloid binds strongly to B-form structures and prevents its conversion to the H^L-form.

As presented above, the kinetic analysis shows that the presence of sanguinarine inhibits both the rate and extent of the B to Z transition (Table 1). In the presence of the small inputs of the alkaloid (lower values of r), the transition was slow (Fig. 6a) while higher ratios completely inhibited the conformational transition (curve 5 in Fig. 6a and curve 2 in Fig. 6c). Moreover the alkaloid converts the Z-form of poly[d(G–C)] and poly[d(G–me^{5}C)] back to the bound B conformation. The semilog plots of absorbance vs. time for poly[d(G–C)] alone and in the presence of identical inputs of sanguinarine and ethidium are presented in Fig. 7. It can be observed that the plots are non-linear even in the case of the drug-free sample, contrary to what is expected for intramolecular, unimolecular process, and this could be due to the length polydispersity of the polynucleotide samples. In the presence of drug, the amount of B-form decreases as the transition proceeds and the differences in affinity of the drug to the two forms results in altered binding densities, which is manifested by the appearance of complex plots. In any case, these plots show a qualitative picture of the DNA binding properties of these two molecules and compare their relative ability to inhibit the B to Z transitions. Apparently, ethidium is a more effective inhibitor of the B to Z transition compared to sanguinarine. In Table 1 the comparative values of the kinetic experiments are presented.

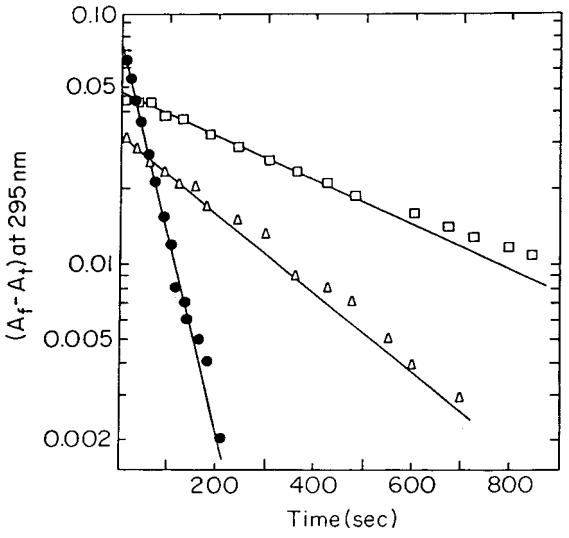

Fig. 7. Semilog plots of the difference in absorbance at time t, $A(t)$ and the final absorbance at 295 nm, $A(f)$, vs. time for poly[d(G–C)] free (●) and in presence of 4.90 μM sanguinarine (△) and 4.90 μM ethidium (□), respectively, at 20°C. The initial rates were calculated from the linear regression of the initial points as shown by the solid lines.

Table 1
Rate and extent of B to Z transition in poly[d(G–C)] in the presence and absence of sanguinarine and ethidium

Ligand	D/P	r[a]	κ[b] (s^{-1})	Extent of relaxation[c] (%)
Sanguinarine	0.00	0	18.3×10^{-3}	100
	0.05	0.041	4.85×10^{-3}	84
	0.10	0.088	3.73×10^{-3}	58
	0.20	0.179	1.54×10^{-3}	21
Ethidium	0.00	0	18.3×10^{-3}	100
	0.10	0.080	2.02×10^{-3}	45

[a] r-Values are calculated from $r = C_B/P$ as described in Section 2.2.
[b] κ is the initial rate constant from the slope of Fig. 7.
[c] The extent of relaxation (%) was calculated as $[(\Delta A^{o}_{295})/(\Delta A^{o}_{295})_{D/P=0}] \times 100$.

3.7. Cooperative binding of sanguinarine to Z and H^L-forms

To understand in more detail the nature of the conformational transitions in CD and the kinetic data, spectrophotometric binding studies of the

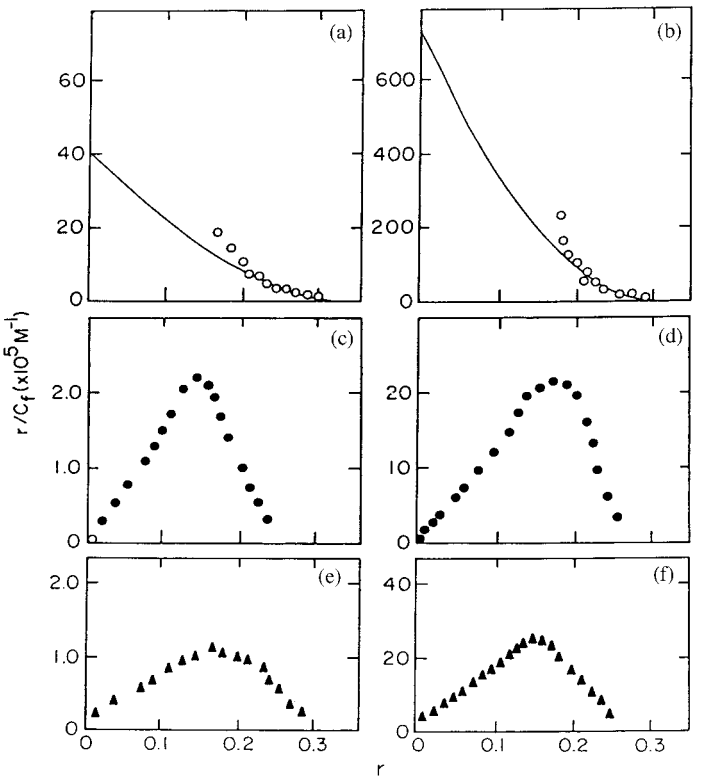

Fig. 8. Equilibrium binding isotherm for the interaction of sanguinarine with poly[d(G–C)] and poly[d(G–me^{5}C)] under B-form, Z-form and H^L-form conditions. Scatchard plots are shown for (a,b) B-form, (c,d) Z-form, and (e,f) H^L-form. The solid lines represent the non-linear least squares best fit of the experimental points analyzed according to the methods of McGhee and von Hippel [59]. The points in the Scatchard plot in (c,d) and in (e,f) show a cooperative type of binding for Z-form and H^L-form, respectively.

alkaloid with the three forms were performed. The Scatchard plot for the binding of sanguinarine to the B, Z and H^L-forms of poly[d(G–C)] and poly[d(G–me^{5}C)] are depicted in Fig. 8. There are remarkable differences in the nature of these plots. In Fig. 8a,b, the Scatchard plots of binding of alkaloid to the B-conformation of these polymers indicate that the isotherms show a positive slope at low r values. This is indicative of a non-cooperative type of binding of sanguinarine to the B-conformation. In Fig. 8c,d, the Scatchard isotherms for the binding to the left-handed Z-conformation are depicted. These curves show a change from a positive to a negative slope in the region $r = 0.15$ and below, indicating positive cooperativity in the binding reactions. With the H^L-form, again the isotherms show a change of the slope (Fig. 8e,f) with both polymers suggesting positive co-operativity in the binding process.

The non-cooperative B-form binding data was analyzed by neighbour exclusion model of McGhee and von Hippel [59]. The best fit value of the binding parameters K' and n calculated from Eq. (5) used to construct the solid lines in

Table 2
Binding parameters for the complexation of sanguinarine and ethidium with B-form structures[a]

Ligand	Polymer	$K' \times 10^{-5}$ (M^{-1})	n
Sanguinarine	Poly[d(G–C)]	40.0 ± 1.8	2.95 ± 0.06
Sanguinarine	Poly[d(G–me^{5}C)]	700 ± 3.2	3.55 ± 0.12
Ethidium	Poly[d(G–C)]	9.00 ± 0.4	3.05 ± 0.10

[a] Five determinations each.

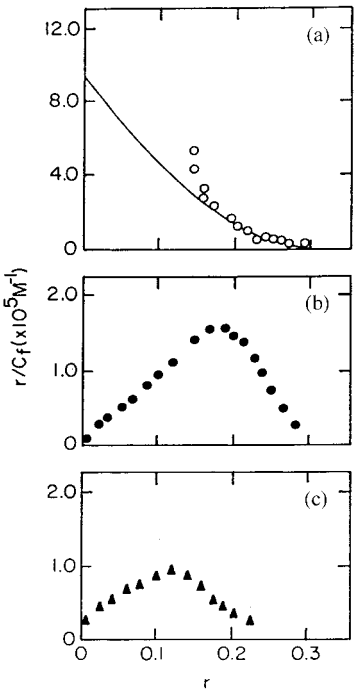

Fig. 9. Scatchard plots for the equilibrium binding of ethidium to B-, Z- and H^L-conformations of poly[d(G–C)]. The plots are shown in (a), (b) and (c) for B-form, Z-form and H^L-form, respectively. The solid line in panel A represents the non-linear least squares best fit of the experimental points to the neighbour exclusion model [59]. The points in (b) and (c) show a cooperative type of binding for Z-form and H^L-form, respectively.

Fig. 8a,b are presented in Table 2 and these data indicate that K' of B-form poly[d(G–me^5C)] is approximately 20 times higher than that of the B-form poly[d(G–C)].

3.8. Comparative binding of ethidium to B, Z and H^L-forms of poly[d(G–C)]

The spectrophotometric binding studies of ethidium with the three conformations of poly[d(G–C)] was performed and the Scatchard plots of these studies are depicted in Fig. 9. The data shows (Fig. 9a) positive slope at lower values of r indicating non-cooperative binding characteristics of ethidium with the B-form structure. The affinity of ethidium with the B-form as derived from the analysis of the data shows a value of 9×10^5 M^{-1} (Table 2). In Fig. 9b, the binding curve shows a change from a positive to a negative slope at $r = 0.19$ and below for Z-form structure, while for H^L-form structure same nature of slope appears at $r = 0.11$ and below (Fig. 9c). These plots clearly indicate cooperative type of interaction with the Z- and H^L-form structures.

4. Discussion

Results obtained previously [45–52] have indicated that sanguinarine iminium form intercalates to DNA as evidenced from: hypochromism and bathochromism of the absorption band; quenching of the steady state fluorescence intensity; increase in fluorescence anisotropy, increase in negative and positive ellipticity of DNA; sign and magnitude of the thermodynamic parameters; stabilization of the DNA against thermal denaturation; the increase in the contour length of sonicated rod-like DNA; and induction of unwinding rewinding process of covalently closed superhelical DNA. Further studies [47–50] on the molecular nature of the specificity of sanguinarine towards DNAs of varying base composition and sequence indicated that the binding affinity of sanguinarine is higher for GC-rich DNA than that for AT-rich DNA with considerable specificity towards alternating GC polymer. The intermolecular interaction of sanguinarine at the intercalation site of natural DNAs and homo- and heteropolymer of AT is characterised by negative enthalpy changes and positive entropy changes, while binding to homo- and heteropolymer of GC is reflected by both negative enthalpy changes and entropy changes [51]. To investigate the molecular nature of the interaction with three polymorphic forms, namely B, Z and H^L form of poly[d(G–C)] and poly[d(G–me^5C)], in the present study we have carried out a series of physico-chemical measurements.

4.1. B-Form DNA–sanguinarine complexation

The results presented here show that sanguinarine has a strong affinity towards the B-form poly[d(G–me^5C)] when compared with the B-form poly[d(G–C)] polymer. Binding analysis from

spectrophotometric data indicates that sanguinarine binds both the B-form polymers in a non-cooperative manner. The binding constants as denoted by the values of K' are approximately 20 times higher for poly[d(G−me^{5}C)] polymer clearly showing a higher effective binding to the methylated GC polymer as compared to the GC polymer (Table 2), while the number of occluded sites (n) are more or less the same. These data support our previous results obtained on the complexation of sanguinarine with GC polymer [51]. This high binding affinity is further evidenced from the CD data (Fig. 3) which provide an independent measure of the binding of sanguinarine to the B-form structures. The CD spectral characteristics are similar to those reported with other intercalators namely actinomycin D [62], aristololactam-β-D-glucoside [56,57], elsamycin A [64], etc., and are in agreement with our earlier observations [46,47]. The nature of the perturbation of the CD bands of poly[d(G−me^{5}C)] is similar as that of poly[d(G−C)], but the saturation value attained by the GC polymer ($r = 0.280$) is slightly higher than that of the methylated GC polymer ($r = 0.256$). Binding studies of ethidium to poly[d(G−C)] indicate that the binding constant K' is four times lower compared to sanguinarine under identical experimental conditions. The value of binding constant K' (9×10^5 M^{-1}) for ethidium is in good agreement with the data reported by Bresloff and Crothers [65]. The CD spectral data suggests that the possibility of a helix to helix conformational transition from a B-form structure to right-handed helical form (but not a 'B'-form since the duplex is distorted to accommodate the ligand) upon binding of sanguinarine or ethidium to these two polymers. The difference in CD spectral characteristics can be accounted for by the difference in asymmetry induced in the B-form architecture of each polymer [66] upon binding of the ligands.

4.2. Z-Form DNA–sanguinarine interaction

Poly[d(G−C)] and poly[d(G−me^{5}C)] are known to be the ideal models for studying the B to Z equilibrium transformation under varieties of environmental conditions [2,4]. The alternating GC and its methylated analogue assume left-handed conformation (Z-form) in 4.4 M and 3.0 M NaCl, respectively. Sanguinarine interaction with this high salt Z-form could not be studied as sanguinarine aggregates under these high salt conditions as evidenced from CD and absorption studies. However, in order to provide a detailed analysis of interaction of sanguinarine to low salt induced Z-form structure, 40 μM and 20 μM $[Co(NH_3)_6]Cl_3$ was used to stabilize the left-handed conformation (Z-form) of poly[d(G−C)] and poly[d(G−me^{5}C)] structure, respectively. In solution, the formation of negative CD around 295 nm is considered to be the diagnostic feature of isomerization of these polymers to the left-handed Z-form [2,4]. The right-handed B-DNA and the left-handed Z-DNA represent two substantially different structural motifs that are in equilibrium with each other. The CD spectral changes (Figs. 3 and 4) indicate a direct conversion of the Z-form towards the characteristic right-handed bound complex without forming any intermediate forms. The CD spectral data are similar to those obtained with other DNA binding ligands [64,67] and were used to infer that the local environment of the polynucleotide at the binding site is a right-handed helical conformation [62–64,68]. For daunomycin it has been reported [68] that the energetically unfavourable Z to right-handed transition, at high salt, is driven by coupling to the energetically favourable binding of daunomycin to the right-handed helical conformation. Our comparative studies with ethidium the classical intercalator testifies to this argument (Fig. 4), where under similar buffer conditions a true conversion to the DNA-bound right-handed form occurs and this is in agreement with several other reports [62–64,68]. The ellipticity changes in the 295-nm band is used to deduce the number of base pairs converted to the right-handed helical form of bound sanguinarine as demonstrated in several other cases like elsamycin A [64], ethidium [67] and daunomycin [68]. These results (Fig. 5) indicate that in our experimental conditions, 5–6 nucleotides (i.e. 2–3 bp) of the left-handed Z-form are converted to bound right-handed helical form by sanguinarine. Similarly our CD analysis of ethidium data also

suggests the conversion of 2–3 bp of the Z-form to the right-handed helical form. In earlier reports Walker et al. [63] have observed that ethidium could convert 25 bp from Z-form to right-handed form under conditions of 40 μM $[Co(NH_3)_6]Cl_3$ in low salt buffer at pH 8.0. At the same time in high salt solution ethidium converted only 3–4 bp of the Z-form of poly[d(G–C)] to bound right-handed form [63]. We have performed similar experiments with ethidium under conditions of pH 8.0 and have observed that ethidium can convert 25 bp of the Z-form of poly[d(G–C)] to bound right-handed helical form. This comparative study at pH 8.0 and 5.2 clearly underscores the influence of pH on the number of base pairs being switched from Z-form to bound right-handed helical form. The DNA binding iminium ion of sanguinarine exists only at pH 5.2 enabling us to do all experiments at this pH. The results presented in Figs. 6 and 7 demonstrate that sanguinarine inhibits both the rate and extent of the B to Z transition of poly[d(G–C)] and poly[d(G–me^{5}C)]. It is interesting to note that at identical alkaloid/DNA molar ratio the rate of Z to B conversion is somewhat slower for sanguinarine than for ethidium (Table 1). Gilbert et al. [69] reported that the size of the ligand is a crucial determinant of its efficiency as an inhibitor of the B to Z transition, these small differences might be a direct consequence of the smaller size of sanguinarine as the ethidium molecule is imposed of the planar tricycle phenanthrinidium ring and a secondary phenyl group perpendicular to the primary ring system compared to sanguinarine. We have used both kinetics and CD experiments to determine the tranformation of Z-form to bound right-handed form as a function of r (Fig. 5 and Table 2). We have found that there is a clear coincidence between our experimental data and those described elsewhere for different compounds showing how an energetically unfavourable Z to bound right-handed helical transition can be driven by coupling it to the energetically favourable interaction of sanguinarine with the B-form DNA. The spectrophotometric binding data of sanguinarine to Z-form is cooperative in nature as demonstrated in Fig. 8. Comparative studies with ethidium also present similar picture (Fig. 9). Ethidium [63,67], actinomycin D [62], daunomycin [68] and elsamycin A [64] have shown cooperative binding effects similar to those presented in Fig. 8 for sanguinarine while proflavine binding does not show such a cooperative behaviour [70]. The results presented in Figs. 5 and 8 show close similarity to the previous studies on the interaction of ethidium [67] and daunomycin [68] with poly[d(G–C)] and poly[d(G–me^{5}C)] where they fit to the allosteric model of Crothers and coworkers [65]. Our data indicate that the transition occurred would follow an allosteric model at stoichiometries of approximately one sanguinarine molecule per 2–3 bp and one ethidium molecule per 2 bp. The CD results also suggest that a right-handed bound conformation is formed at the intercalation site. Thus, it is likely that the conversion of the polynucleotides from a Z-form to a right-handed bound conformation is sequential one, so neither sanguinarine nor ethidium does need to bind to the Z-form DNA.

Gene expression depends upon interaction between nucleic acids and regulatory proteins. These interactions are of high specificity [71]. Similarly, compounds like antitumour drugs that may interfere with genetic expression should present these properties. Nevertheless, it is noteworthy that at least some drugs might effectively interfere with DNA–protein interaction and therefore, play an important role in modulating the gene expression [72,73]. Many such drugs appear to have a remarkable effect on the B to Z transition [62–70]. Studies on the effect of intercalating drugs on the rate of B to Z transition shows that actinomycin D is more effective than ethidium as an inhibitor of the transition [74], while the relative efficiency of ethidium and daunomycin in inhibiting such conformational change is identical [62–64]. Our results indicate that ethidium is slightly more efficient than sanguinarine in inhibiting B to Z transition but, at the same time, the latter has higher binding affinity to the B-form.

Left-handed DNA was demonstrated to exist in *Escherichia coli* [75] on the basis of the observation that in vitro a target site is not methylated by

its specific methylase when the site is nearer [76] or in a left-handed Z helix [77,78]. However, these enzymes do act on the same target sequences when they exist in a right-handed B structure. The gene for a temperature sensitive *Eco*RI methylase was cloned in a pACYC184 derivative to give pRW1602, and conditions were established for its temperature-dependent expression [75]. pRW1602 was co-transformed with any one of 10 pBR322 derivative plasmids that contained inserts with different capabilities in vitro of forming Z structures, depending on the lengths, orientations and types of sequences. The excellent correlations between the ability of a sequence to inhibit methylation by *Eco*RI methylase in vivo and in vitro, as well as its relative capacity to adopt a Z helix in vitro, established the concept of left-handed DNA in vivo. Unlike B-DNA, left-handed Z-DNA is highly immunogenic. Antibodies have been produced, both polyclonal [79] and monoclonal [80], which bind specifically to Z-DNA. These have been used to demonstrate the existence of Z-DNA in various biological systems [9,11]. While the precise biological functions of Z-DNA are yet to be identified, its role in regulating DNA supercoiling has been amply demonstrated [5,81]. A recent study by Rich and co-workers [82] has shown that chicken double-stranded RNA adenosine deaminase has strong Z-DNA binding properties. This enzyme is known to work near the transcription apparatus, where a high negative supercoiling density along the DNA chain exists in front of the site of polymerase action [83]. Sanguinarine binds to GC sequences by intercalation [45–52] and it is an effective inhibitor of the B to Z transition and an effector of the Z to B conformational changes. Thus, our results suggest a potential mechanism by which the alkaloid may convey a specific meaning for its regulatory role in biological system.

4.3. H^L-Form DNA–sanguinarine interaction

Protonation of DNA has been studied for several years and it has been suggested that protonation leads to conformational changes before acid denaturation [15–18]. There are considerable evidences in favour of left-handed (Z-like) conformation in poly[d(G–C)] and poly[d(G–me^{5}C)] on protonation [15]. Spectroscopic studies of Marck et al. [16] suggested formation of Hoogsteen base pairs which presumably results from the protonation of the N^7 position of guanine bases and subsequent swing to *syn*-conformation to share the proton with the N^3 of cytosine in GC polymers. Extensive studies were performed in our laboratory on the protonation of natural and synthetic DNAs of varying base composition and sequence of base pairs using absorption thermal melting and CD methods under various environmental conditions [21–25]. The results suggested that the right-handed B-conformation is remarkably perturbed on protonation due to the formation of left-handed conformation. The duplex nature of this unique left-handed conformation was established from thermal melting studies where cooperative helix to coil transition was observed and a new model of Hoogsteen base paired left-handed duplex structure (H^L-form) was proposed under the influence of low pH and low temperature [25]. This structural model was subsequently confirmed by FTIR [26] and Raman spectral measurements [27].

Our results also demonstrate that poly[d(G–C)] and poly[d(G–me^{5}C)] structures are the ideal model for B to H^L equilibrium transformation (Fig. 2). In solution at pH 3.4 and at 10°C, the formation of a characteristic negative CD band around 300 nm in these polymers (Fig. 2) is considered to be the diagnostic feature for isomerization of the polymers to the left-handed H^L conformation. These data are in conformity to our earlier observations [25]. This conformational isomerization under the influence of low pH exhibits an isoelliptic point with the B-form indicating that two conformations of the polymers co-exist under these experimental conditions. Further support to the left-handed type helical conformation under the influence of low pH comes from the absorbance data, where hypochromic effect of the band maximum followed by a hyperchromic effect around 280 nm and an isosbestic point similar to the B to Z isomerization was seen (Fig. 2).

The CD spectra presented in Fig. 3e,f show the nature of the changes on binding of sanguinarine

to H^L-form of poly[d(G–C)] and poly[d(G–me^{5}C)] structures. The results indicate a direct conversion of H^L-form towards the bound right-handed helical form in the presence of sanguinarine. Our comparative studies with ethidium also testify this argument (Fig. 4e,f). However, it is noteworthy that there are significant differences in the CD spectral changes in the H^L-form–ethidium interaction in both polymers. The CD spectral changes, although reminiscent towards bound right-handed conformation, exhibited ellipticity values lower than that seen with the bound form of ethidium with B-form and Z-form structures. These differences can probably be only due to the differences in the asymmetry induced in the complex formation upon binding of ligands under these conditions. It is pertinent to observe that the chemical structures of sanguinarine and ethidium does not undergo any change at pH 3.4. The ellipticity changes at 300 nm used to deduce the number of base pairs converted from H^L form to the bound right-handed form (B-form DNA–sanguinarine complex), indicate that in our experimental conditions approximately 2–3 bp of the H^L-form are converted to the bound right-handed B-form. Similarly, our CD analysis of the ethidium data also suggest the conversion of same number of base pairs of H^L-form to the bound right-handed B-form. Again the spectrophotometric titration data on binding of sanguinarine and ethidium to H^L-form is cooperative with both these polymers. These results can be explained by utilizing the allosteric model of Crothers and coworkers [65] as ethidium has been reported to show allosteric effects in Z to B transitions. Our comparative studies suggest that sanguinarine behaves almost in identical fashion with ethidium. Thus, this suggests that an allosteric model could satisfactorily describe the results of H^L-form–sanguinarine or ethidium interactions. It has also been observed that the conversion of B-form poly[d(G–C)] and poly[d(G–me^{5}C)] to H^L-form is very fast (< 1 s) and it was not possible to detect whether sanguinarine or ethidium could inhibit the rate and extent of formation H^L-form from B-form structure. Sanguinarine does not bind to H^L-form rather it converts H^L-form to the bound right-handed form. Thus, the results show that the energetically unfavourable H^L-form to B-form transition at low pH is driven by coupling to the energetically favourable binding of sanguinarine to the right-handed conformation.

It has been observed that in some biological systems, certain biological functions are relatively favoured at low pH. A pH-dependent enhancement of DNA binding by the ultra bithorax homeodomain has been studied by Li et al. [28]. This report highlights on the relevance of the protonation induced conformational study of nucleic acids with respect to biological systems. Robert-Nicoud et al. [19] had studied the polytene chromosome isolated from the salivary gland of *Chironomus thummi* larvae and its related immunological detection of left-handed Z-DNA. They found that the anti Z-DNA antibody binding to the above is enhanced when DNA is exposed to low pH as revealed from the dramatic increase in the immunofluorescence intensities of the Z-DNA immunoglobulin. Another study of Pozzi et al. [29] has clearly shown that the fusion of Epstein–Barr virus with Raji (lymphoblastoid) cells at low pH is significantly enhanced compared to that at neutral pH. The survival of *Helicobactor pylori*, the major aetiological factor in chronic gastritis in human at low pH environment is probably related to its pathogenicity [30]. Foster [31] has recently reported studies on the low pH adaptation and tolerance response (ATR) of *Salmonella typhimurium* and has found that the key to ATR is the synthesis of a series of acid shock inducible proteins for log and stationary phase of ATR. These studies coupled with earlier reports by Hicky and Hirshfield [32] have drawn considerable attention to pH regulated gene expression and related environmental influences over DNA topology and subsequent effect on selected gene expression [31]. The influences over DNA topology of pH regulated genes is thus of particular interest as it involves a great deal of structural aspects of DNA correlated to its functions. A recent Raman spectroscopic study of low-pH-induced changes in DNA structure of polytene chromosomes reveals that left-handed-like DNA structure is present at low pH [33]. Thus, as DNA undergoes defined conformational change

under the influence of low pH, the ability of this conformational status to interact with sanguinarine may convey some specified meaning for its regulatory role in biological systems.

5. Conclusion

To conclude, our studies show that the benzophenanthridine alkaloid, sanguinarine exhibits a remarkable affinity for the B-form structure of poly[d(G–C)] and poly[d(G–me^{5}C)] and the binding process is non-cooperative in nature. It also converts cooperatively the left-handed Z-form and the left-handed H^L-form of these polynucleotides to bound right-handed helical form. Sanguinarine inhibits both the rate and extent of B to Z transition. Comparative studies with ethidium suggests that although sanguinarine has a much stronger binding affinity to the B-form DNA structures, kinetically it appears to be less effective in inhibiting the B to Z transitions. If Z DNA plays significant role in the control of the cell processes, our results suggest a potential mechanism by which the alkaloid might inhibit replicative events and gene transcription, probably leading to its usefulness as an antitumour alkaloid.

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