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Protonated Forms of Poly[d(G-C)] and Poly(dG).poly(dC) and Their Interaction with Berberine

Gopinatha Suresh Kumar, Suman Das, Kakali Bhadra and Motilal Maiti*

Biophysical Chemistry Laboratory, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700 032, India

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Abstract—The pH -induced structural changes on the conformation of homo- and hetero-polymers of guanosine–citydine (G.C) sequences were investigated using spectrophotometric and circular dichroic techniques. At pH 3.40, 10 mM [Na^+] and 10 °C both polynucleotides adopted a unique and stable structural conformation different from their respective B-form structures. The protonated hetero-polymer is established as left-handed structure with Hoogsteen base pairing (H^L-form) while the homo-polymer favored Watson–Crick base pairing with different stacking arrangements from that of B-form structure as evident from thermal melting and circular dichroic studies. The interaction of berberine, a naturally occurring protoberberine group of plant alkaloid, with the protonated structures was studied using various biophysical techniques. Binding of berberine to the H^L-form structure resulted in intrinsic circular dichroic changes and generation of extrinsic circular dichroic bands with opposite sign and magnitude compared to its B-form structure while with the homo-polymer of G.C no such reversal of extrinsic circular dichroic bands was seen indicating different stacking arrangement of berberine at the interaction site. Scatchard analysis of the binding data, however, indicated non-cooperative binding to both the protonated forms similar to that of their respective B-form structure. Fluorescence spectral studies, on the other hand, showed remarkable increase in the intrinsic fluorescence of the alkaloid in presence of the protonated forms compared to their respective B-form structure. These results suggest that berberine could be used as a probe to detect the alteration of structural handedness due to protonation and may potentiate its use in regulatory roles for biological functions.

Introduction

It is now well established that structurally DNA is a polymorphic macromolecule that can exist in a variety of conformations.[1] In aqueous solutions at physiological pH, DNA exists as right-handed B-form double helix with Watson–Crick base pairing. In several polynucleotides, however, under a variety of conditions the right handed B-form structure is converted to the left handed Z-form that still retains the Watson–Crick base pairing.[2–5] With convincing evidences, for the existence as well as biological roles for left-handed Z-form in vivo,[6] the importance of polymorphic DNA structures have gained renewed significance for their potential roles in genetic regulatory processes.[7–9]

Protonation induced structural modifications in DNA have been an important area of investigation as pH manifests itself as a powerful and sensitive factor in all biological processes.[11–16] Although there is no general consensus on the precise nature of the changes that occur in DNA as the pH is lowered, it is now accepted that protonation leads to remarkable changes in the DNA conformation that is by and large dependent on the base composition and ionic strength of the medium.[17–24] Base protonation has been suggested to promote B to Z transition in synthetic hetero-guanine-cytosine polynucleotides at low [Na^+] ion concentrations.[25] We had earlier demonstrated from extensive spectroscopic studies that protonation leads to a unique left handed structure with Hoogsteen base pairs (Fig. 1A) designated as H^L-form in DNA at low ionic strength and low temperature.[26–28] High-resolution Raman and FTIR studies[29,30] later confirmed this structural model. This H^L-form structure differs significantly in both handedness and the base-pairing scheme with the B-form structure (Fig. 1B).

Binding of small molecules and drugs to altered DNA structures has been an active area of investigation.[27,28,31–40] The ability of small molecules to interact differentially with different nucleic acid

*Corresponding author. Tel.: +91-33-2472-4049; fax: +91-33-2473-5197; e-mail: mmaiti@iicb.res.in

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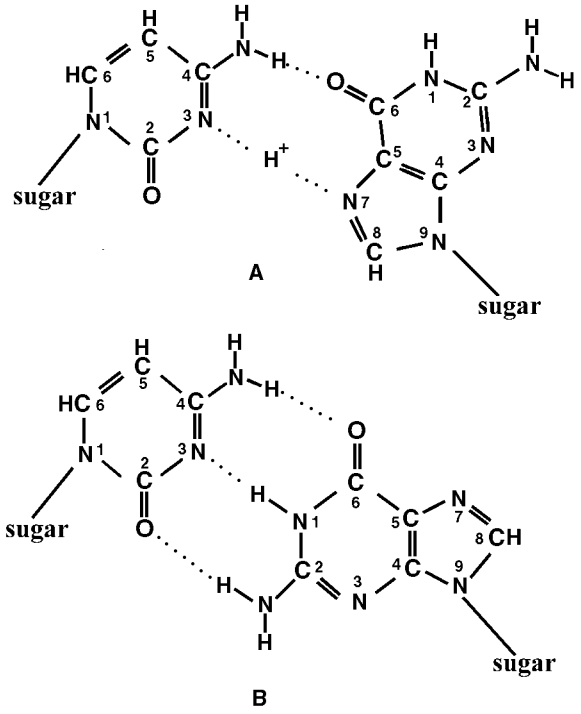

Figure 1. Structure of (A) Hoogsteen and (B) Watson–Crick pairing in the guanosine-cytidine base pair.

conformations will convey specific meaning for their regulatory roles in biological systems. For example, several intercalating molecules have now been characterized to convert the left handed Z-form DNA conformation to the bound B-form conformation. Ethidium,[31,32] actinomycin D,[33] daunomycin,[34] elsamicin A,[35] sanguinarine[27,28] and aristololactam-β-D-glucoside[36] cooperatively convert the Z-form structure back to the bound B-form conformation. The binding of drugs, however, to other polymorphic forms of DNA is still poorly studied.[37,38] Chen had reported[39] that pyrene strongly binds to the DNA at acidic pH. Our studies had demonstrated[27,28] that sanguinarine and ethidium converted the H^L-form structures of the hetero-polymer and its methylated analogue, poly[d(G-^{m5}C)] cooperatively to bound B-form while aristololactam-β-D-glucoside strongly bound the H^L-form structures of these polymers.[40]

This paper is divided into two sections, beginning with a description of the pH dependent conformational transitions in homo- and hetero-polymers of guanosine–cytidine (G.C) followed by their interactions with the benzodioxolo-benzoquinolizine alkaloid berberine. Berberine (Fig. 2) is one of the most widely distributed plant alkaloid belonging to the structural class of protoberberines. Berberine has extensive biological activities.[41–44] It has strong anti mutagenic and anti malarial properties and is cytotoxic towards several tumor cells.[41] Recently, the duel topoisomerase I and II poisoning activities of berberine have been revealed.[45–48] Studies also indicated that berberine induced apoptosis of human leukemia HL-60 cells is associated with down regulation of nuceophosmin/B23 and telomerase activity.[49] DNA binding property of this alkaloid has been studied in our and other laboratories.[45–54] Using extensive spectroscopic and hydrodynamic studies with a variety of B-form DNA's, a partial intercalative mode of interaction for this alkaloid was established.[51–53] We also illustrated an adenosine–thymidine (A.T) base pair specificity from our studies.[51–53] Our partial intercalation model was substantiated further from NMR studies as well.[54] More recent physicochemical and biochemical studies in combination with computer modeling techniques from other laboratories again strongly favored our partial intercalation model, in which the planar benzophenanthridine moiety of the ligand remained intercalated between the base pairs and the rest of the molecule protruded deep into the minor groove of the DNA.[45,46] However, the very recent high-resolution ^{1}H and ^{31}P NMR results on berberine binding to a variety of oligonucleotides favored a groove binding geometry.[55] In this study we investigate the binding of berberine to the H^L-form of the hetero- and protonated form of the homo-polymer of G.C and also their respective B-form structure for comparison through spectroscopic techniques. Our results tend to strongly suggest that berberine can detect the change of handedness of the protonated form as evidenced from the display of extrinsic circular dichroic pattern with opposite sign and magnitude from that with the B-form.

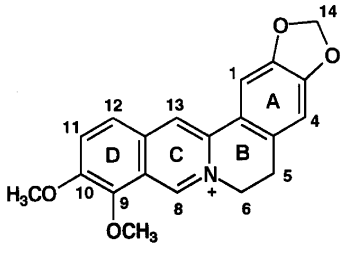

Figure 2. Chemical structure of Berberine.

Results

CD and UV spectral changes of hetero- and homo-polymer of G.C under the influence of pH

The circular dichroic changes of poly[d(G-C)] and poly(dG).poly(dC) at different pH values between 7.0 and 3.0 at 10 mM [Na^+] at 10±1 °C are presented in Figure 3. In the hetero-polymer, the changes are manifested as an initial blue shift of the positive CD band, decrease in the intensity of the 250 nm negative band resulting in the formation of a well defined negative band around 300 nm. The protonated spectrum that remained invariant in the pH range 3.40–3.10 is characterized by a negative band in the 300 nm region and a positive band in the 260 nm region followed by the negative band around 240 nm (spectrum 5 in Fig. 3A). The series of spectra exhibited an isoelliptic point around 285 nm characterizing equilibrium between in the conformational rearrangement. The UV spectral changes accompanying the CD changes in this polymer are depicted in the inset of Figure 3A. The changes essentially involve a hypochromic effect in the 260 nm

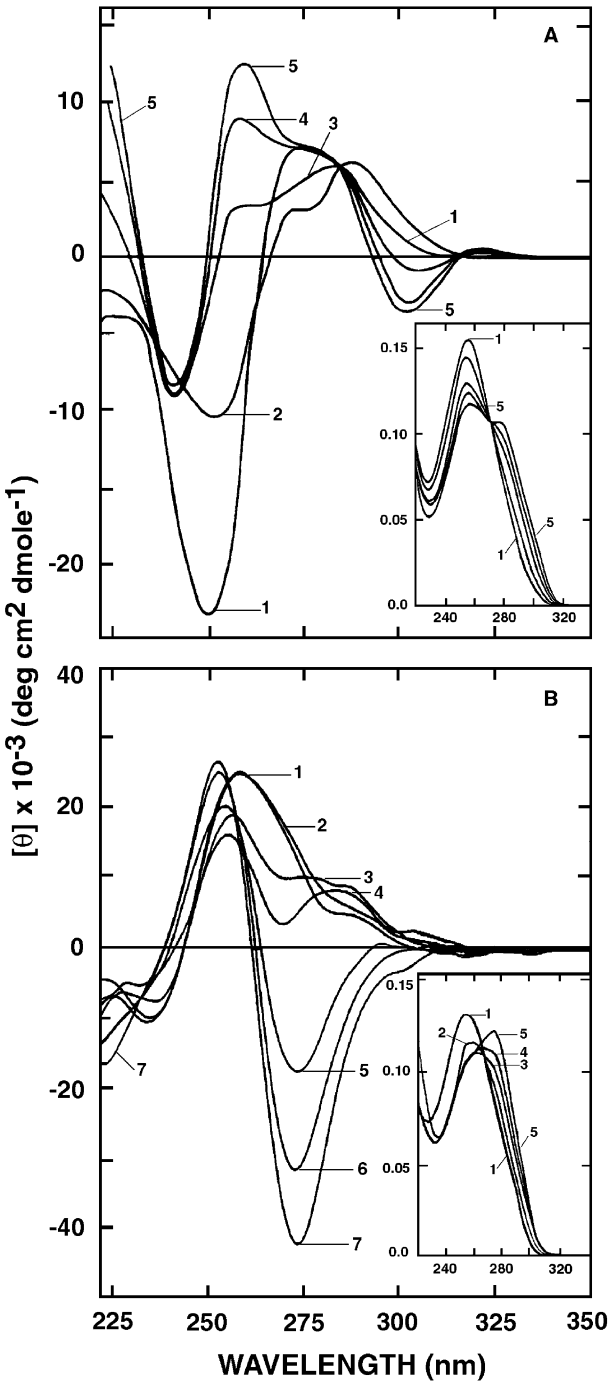

Figure 3. Influence of pH on the CD spectra of (A) poly[d(G-C)] and (B) poly(dG). poly(dC) in citrate–phosphate buffer at 10±1 °C. (A.) Curves 1–5 denote pH values of 7.0, 4.20, 3.80, 3.65 and 3.40 and (B) curves 1–7 denote pH values 7.0, 5.90, 4.70, 4.00, 3.80, 3.65 and 3.40, respectively. Inset: UV spectral changes of poly[d(G-C)] and poly(dG). Poly(dC) at different pH values at 10±1 °C. Curves 1–5 in the inset of A denote pH values 7.0–4.90, 4.20, 3.85, 3.65 and 3.40 and curves 1–5 in the inset of B denote pH values of 7.0–5.90, 4.75, 3.80, 3.40 and 3.20, respectively.

band with concomitant enhancement of the extinction in the 280 nm region; the later eventually developed into a band. The H^L-form structure developed in this polymer in the pH range 3.40–3.10 has characteristic UV spectral features (spectrum no. 5 of inset in Fig. 3A) and is in conformity with our earlier observation.[26]

The CD spectral changes in poly(dG).poly(dC) upon reducing the pH was remarkably different from that observed for the hetero-polymer (Fig. 3B). The B-form CD spectrum of this polymer at pH 7.0 is characterized by large positive band in the 260 nm region (spectrum 1, Fig. 3B). As the pH is lowered, a negative band replaces the positive ellipticity in the 250–300 nm region, with maximum centered around 275 nm. The protonated spectrum in the pH range 3.4–3.0 has a strong negative band at 275 nm followed by a positive band around 250 nm with a cross over near the wavelength maximum of the native spectrum (spectrum 7 of Fig. 3B). The UV spectral changes associated with the pH variation (inset of Fig. 3B) is characterized by an initial decrease in absorbance of the 255 nm band, a red shift and an increase in the absorbance. The absorbance spectrum of the protonated form of this polymer has a wavelength maximum around 275 nm (spectrum no. 5 in the inset of Fig. 3B).

Binding of berberine to H^L-form of hetero-polymer and protonated form of homo-polymer of G.C

The characteristic B-form CD spectrum of the hetero-polymer undergoes remarkable changes in presence of berberine manifested by large enhancement of the positive band with little alteration in the negative band (Fig. 4A). The ellipticity of this band increased from an initial value of $\sim$7000 ° cm^2 $dmole^{-1}$ to almost double at the saturation point. This is characteristic feature of the elongation of the duplex as a result of binding of the alkaloid between the base pairs of DNA. In Figure 4B, the binding of the alkaloid to the H^L-form of poly[d(G-C)] is depicted. Here also presence of the alkaloid produced increase in the ellipticity of the positive band with little alterations in the negative band. Concomitant with this increase there was also the development of an extrinsic CD band with positive ellipticity in the 330–400 nm region for the bound alkaloid molecules. A saturation effect in the enhancement of the positive band was achieved with concomitant saturation in the extrinsic CD band. Thus the strong binding of the alkaloid to the H^L-form generated a strong extrinsic CD band and this was not observed with the binding to B-form structure (Fig. 4A).

In Figure 4C and D, the interaction of the alkaloid to the B and protonated forms of the homo-polymer is depicted. The changes observed on progressive increase of berberine concentration with the B-form polymer was marginal (Fig. 4C) with no indication of the development of any extrinsic CD bands. In Figure 4D, the CD spectral changes on the interaction of berberine to the protonated structure of poly(dG).poly(dC) show remarkable changes on binding. The 275 nm negative band decreased in ellipticity rapidly and crossed over to the positive side to produce a strong positive band around 280 nm. Concomitant with this change the 250 nm positive band of the protonated poly(dG).poly(dC) decreased and disappeared. The fully berberine bound spectrum of protonated poly(dG).poly(dC) has a single positive band around 280 nm with a relatively small band in the 220 nm region (spectrum no. 10 of Fig. 4D). Here

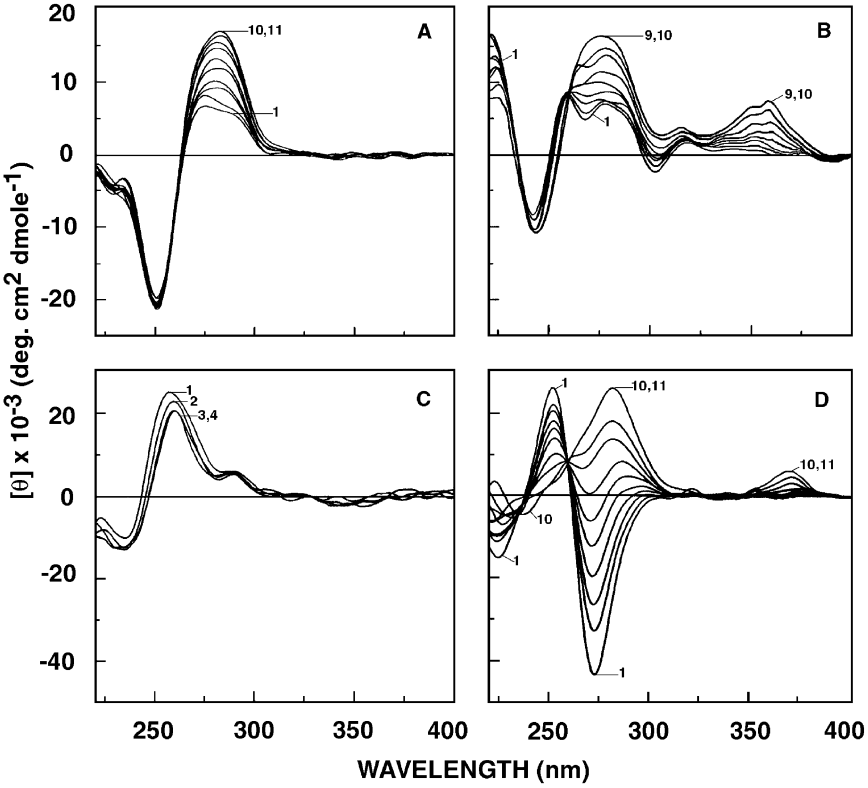

Figure 4. Representative CD spectra resulting from the interaction of berberine with H^L-form poly[d(G-C)], protonated form of poly(dG).poly(dC) and their respective B-form structures at 10 ± 1 °C (A) Curves (1–11) denote B-form poly[d(G-C)] (40 μM) treated with 0, 1.63, 3.67, 6.11, 9.40, 12.60, 15.81, 19.04, 22.24, 25.43 and 28.62 μM of berberine in CP buffer pH 7.0; (B) curves (1–10) denote H^L-form poly[d(G-C)] (40 μM) treated with 0, 6.52, 12.99, 16.22, 19.44, 22.64, 25.83, 29.02, 32.20 and 35.35 μM of berberine in CP buffer pH 3.40; (C) curves (1-4) denote B-form poly(dG). poly(dC) (31.0 μM) treated with 0, 6.51, 11.33 and 17.30 μM of berberine in CP buffer of pH 7.0 and (D) curves (1-11) denote protonated form poly(dG). poly(dC) (31.0 μM) treated with 0, 0.82, 2.0, 3.7, 6.1, 10.1, 14.1, 18.1, 22.0, 26.0 and 30.0 μM of berberine in CP buffer of pH 3.40.

again like in the case with the H^L-form poly[d(G-C)] an extrinsic CD band was developed in the region 340–380 nm. However the extrinsic CD developed was week in intensity compared to that observed with poly[d(G-C)].

Binding of berberine to the Z-form of hetero-polymer of G.C

In order to compare the binding of berberine to Z-form DNA (left-handed Watson–Crick base paired structure) we have studied the interaction of the alkaloid with Z–DNA structures of poly[d(G-C)]. The CD spectra of the Z-form poly[d(G-C)] remained invariant in presence of berberine up to a P/D (nucleotide phosphate/drug) of ≤ 0.80 (figure. not shown) indicating clearly that berberine neither bound to Z-form structures nor it altered the Z-form structure to bound B-form.

Extrinsic CD studies of berberine with H^L-form of hetero-polymer and protonated form of homo-polymer of G.C

To understand in more details the binding of berberine to the two-protonated forms, extrinsic CD measurements were performed in the region (300–500 nm) keeping a fixed concentration of the alkaloid and varying the concentration of the polynucleotides. In order to compare the data under identical conditions the interaction of berberine with their respective B-form structure was also studied (Fig. 5). Binding of berberine to both B-form and protonated form generated extrinsic CD bands, but the nature and extent were different. In Figure 5A and B the extrinsic CD observed with the B and H^L-forms of poly[d(G-C)] are presented. With the B-form- berberine complex, there was a broad CD band in the 400–500 nm region with maximum around 435 nm followed by an exciton split type band in the 340 nm region at high P/D ratios (≥ 10). On decreasing the P/D ratio there were drastic changes in the 340 nm region resulting in the development of a positive band that further increased, reached a maximum and then decreased in intensity. The 435 nm band however decreased in ellipticity with decreasing polymer concentration. In Figure 5B, the extrinsic CD changes observed for H^L-form-berberine complex is presented. The association of berberine molecules to the H^L-form induces remarkably strong positive optical activity in the 300–400 nm region followed by a negative band in the 400–500 nm region. This is almost opposite in magnitude and shape to that observed with the B-form structure (Fig. 5A) at the same P/D ratio. As the P/D decreased, the ellipticity of the positive and the negative bands reduced in intensity.

The CD changes seen on the interaction of berberine with the B- and protonated forms of the homo-polymer

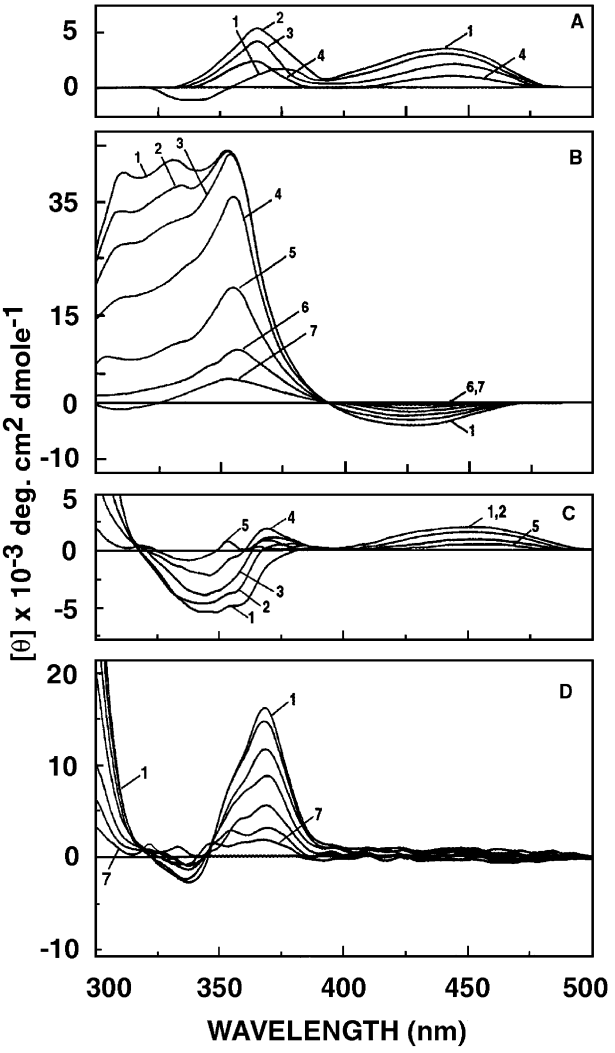

Figure 5. Extrinsic CD spectra of berberine (50 μM) at 10±1 °C treated with (A) B-form poly[d(G-C)] of concentrations 500, 143, 100 and 50 μM as shown by curves 1–4, (B) H^L-form poly[d(G-C)] of concentrations 500, 400, 300, 200, 100, 50 and 25 μM as shown by curves 1–7, (C) B-form poly(dG).poly(dC) of concentrations 500, 300, 200, 100 and 50 μM as shown by curves 1–5 and (D) protonated form poly(dG).poly(dC) of concentrations 500, 400, 300, 200, 100, 50 and 25 μM as shown by curves 1–7, respectively. A complex of protonated DNA and berberine or B-form DNA and berberine at P/D = 10 was made and this complex was diluted using the respective buffer containing 50 μM of berberine in each dilution in order to get decreasing P/D values. The expressed molar ellipticity is based on the alkaloid concentrations.

are presented in Figure 5C and D, respectively. With the B-form, a positive CD is generated in the 400–500 nm region, but the intensity is relatively weak compared to that with the hetero-polymer. In the 300–400 nm regions the nature of the extrinsic CD observed here is also markedly different. A negative CD band (curve 1, Fig. 5C) was developed at high DNA concentrations (P/D = 10) that reduced in intensity as the P/D decreased. On the other hand the protonated form of the homo-polymer (Fig. 5D) produced a large positive band with maximum around 370 nm that decreased in ellipticity as the P/D decreased. Significantly, there was no CD band in the 400–500 nm region at any P/D ratio with this polymer.

Absorption spectral study

In order to understand the nature of the conformational changes observed in CD on binding of berberine, spectrophotometric binding studies of the alkaloid with the two forms of the polymers were performed under appropriate solution conditions (Fig. 6). Presence of increasing concentrations of both the B-form polymers (Fig. 6A and C) resulted in typical hypochromic and bathochromic effects in the visible absorption spectrum of berberine. The characteristic isosbestic points in the series of spectra indicated clearly an equilibrium in the binding phenomenon. In the case of protonated forms (Fig. 6B and D) the pronounced changes were significantly lower; the least changes being observed in case of protonated (H^L) form of the hetero-polymer (Fig. 6B).

Binding isotherms for each case is nonlinear and concave upward, involving more than one type of binding site. Since we found no sign of sigmoidal behavior for the occurrence of cooperativity in our system, we adopted the neighbour exclusion model[56] using the SCATPLOT program[57] for non-cooperative binding phenomenon to fit our experimental data. We have also found that such a model adequately fits our data within the regions of Scatchard plot corresponding to the range 30% (lower) to 90% (upper) of each protonated and B-form polymer. Representative binding isotherms are illustrated in the inset of Figure 6. The binding affinity of berberine to the B and protonated forms of both polymers is depicted in Table 1. The data reveals that the alkaloid has higher affinity for the B-form structures compared to the protonated forms.

Thermal melting study

Under the conditions of our study, the B-form of poly[d(G-C)] denatured at a temperature higher than 100 °C. Therefore the stabilization of the B-from structure in presence of berberine could not be monitored. The protonated form of poly[d(G-C)] exhibited a melting temperature of 40 °C. In presence of berberine (D/P = 0.6) a stabilization of 5 °C was achieved without any significant change in the co-operativity of the transition. The B-form of the homo-polymer exhibited a helix to coil transition with a T_m 80 °C. In presence of berberine (D/P = 0.6) an enhancement of 3 °C was observed in the homo-polymer. The protonated form of the homo-polymer did not exhibit any melting up to 100 °C. Comparative melting data is also presented in Table 1.

Fluorescence spectral study

In aqueous buffer, binding to B-form DNA enhances the intrinsic fluorescence of berberine.[51,52] In Figure 7, comparative fluorescence spectral data on the interaction of berberine with the B and protonated forms of the two polymers are presented. The protonated structures enhanced the fluorescence of berberine several folds (Fig. 7B and D) compared to the B-form structures (Fig. 7A and C) suggesting differences in the nature of the binding sites of the alkaloid on these

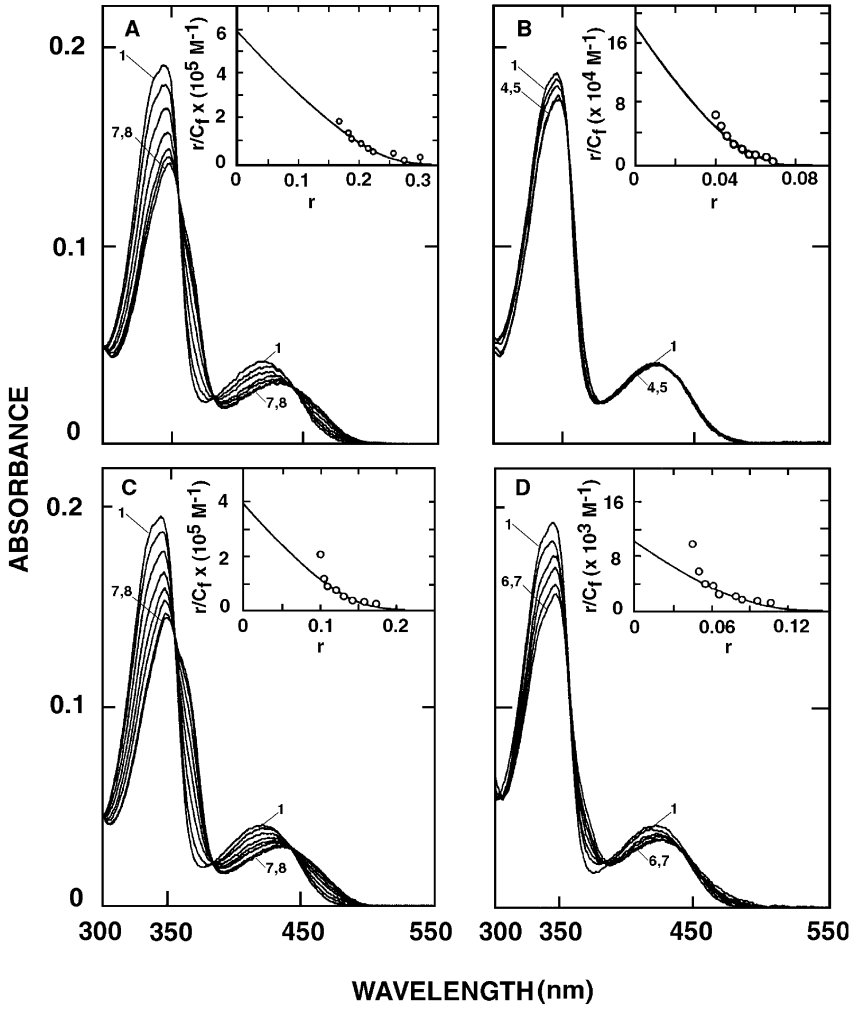

Figure 6. Absorption spectra of berberine (8.3 μM, curve 1) treated with B-form and H^L-form of poly[d(G-C)] (A and B) and protonated form of poly(dG).poly(dC) (C and D) at 10±1 °C. (A) Curves 2–8 denote 5.32, 13.30, 22.91, 34.48, 42.40, 50.28 and 58.15 μM; (B) curves 2–5 denote 33.20, 66.40, 91.30 and 124.5 μM; (C) curves 2–8 denote 9.98, 19.92, 31.79, 45.58, 61.24, 82.61 and 94.19 μM and (D) curves 2–7 denote 16.28, 32.57, 50.0, 81.65, 122.47 and 163.3 μM. Inset: representative Scatchard plots for the binding of berberine to (A) B-form poly[d(G-C)] (B) H^L-form poly[d(G-C)], (C) B-form poly(dG).poly(dC) and (D) protonated form of poly(dG).poly(dC). The solid lines represent the non-linear least square best fit of the experimental points to the neighbor exclusion model (eq. 1) obtained using the computer program SCATPLOT.[57] This model adequately fits the data within the regions of the Scatchard plot ranging from 30% (lower limit) and 90% (upper limit). Values of K' and n are presented in Table 1.

Table 1. Binding parameters and T_m data on the interactions of berberine with B-form and H^L-form of poly[d(G-C)] and protonated form of poly(dG).poly(dC)[a]

Polymer	Conformation	K' ($\times 10^{-5}$)	n	ΔT_m[d]
Poly[d(G-C)]	B-form[b]	5.8	3.1	n.d
Poly[d(G-C)]	H^L-form[c]	1.8	11.3	5.0
Poly(dG).Poly(dC)	B-form	3.9	4.7	3.0
Poly(dG).poly(dC)	Protonated form[c]	0.1	6.8	No melting up to 100 °C

[a]Average of three determinations.
[b]In 10 mM citrate–phosphate buffer, pH 7.0.
[c]In 10 mM citrate–phosphate buffer, pH 3.40.
[d]ΔT_m is the [T_m of drug–DNA complex $-T_m$ of DNA]; T_m of poly[d(G-C)] in the H^L-form and the T_m of poly(dG).poly(dC) in the B-form were 40 was 86 °C, respectively.

structures. The maximum enhancement was observed with the protonated form of poly[d(G-C)] (Fig. 7B).

Discussion

Results obtained previously from extensive spectroscopic and viscometric studies have revealed that berberine forms a molecular complex with DNA.[50–55] Our studies have unequivocally characterized berberine–B-form DNA complexation to be partially intercalating[52,53] and was further supported by biochemical and molecular modeling studies by other workers.[45–47] In the present study we have chosen to compare the interaction of berberine with B-form and protonated structures of the hetero and the homo-polymer of G.C

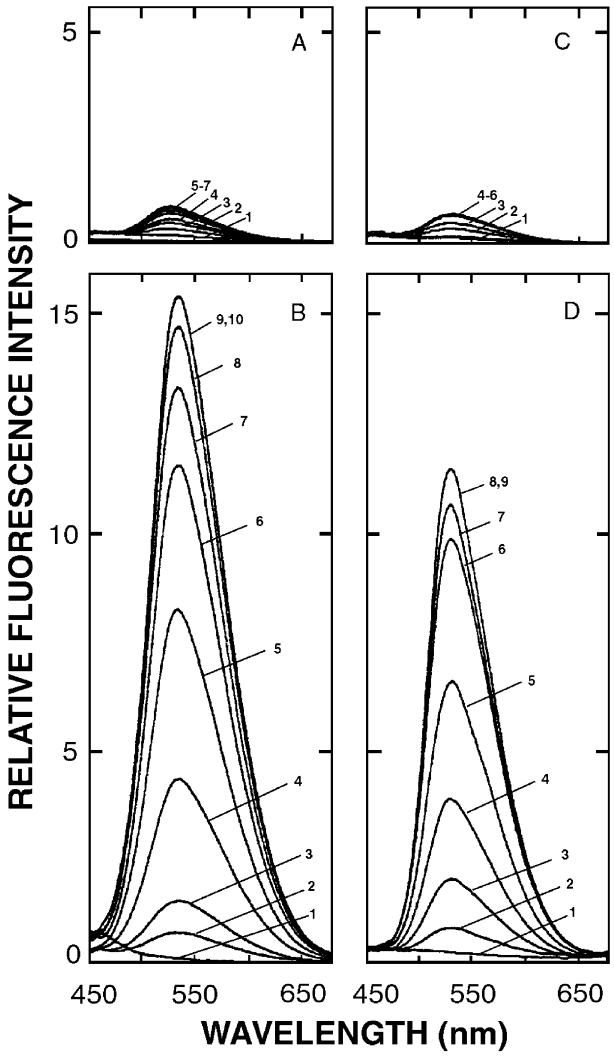

Figure 7. Fluorescence emission spectra of berberine (3.1 μM) (curve 1) at 10±1 °C treated with B-form and H^L-form of poly[d(G-C)] and B-form and protonated form of poly(dG).poly(dC). (A) Curves 2–7 denote 7.4, 23.0, 36.8, 51.4, 66.0 and 81.0 μM of B-form poly[d(G-C)]; (B) curves 2–10 denote 6.2, 15.5, 30.0, 55.0, 80.0, 100.0, 125.0, 138.0 and 160.0 μM of H^L-form poly[d(G-C)]; (C) curves 2–6 denote 13.0, 25.5, 45.2, 70.0 and 96.0 μM of B-form poly(dG).poly(dC); and (D) curves 2–9 denote 7.0, 16.5, 35.0, 59, 82, 110, 131, 152 and 181 μM of protonated form of poly(dG).poly(dC).

sequences. In the G.C homo-polymer, the CD changes on protonation were markedly different from that observed with the G.C hetero-polymer. Several earlier studies had focused on the protonation of G.C base pairs in homo-polymer, but the exact nature of the changes remained obscure.[58,59] Polypurine–polypyrimidine sequences with high cytosine content have been found to be located in abundance in regions of regulatory importance[60] and some of these regions have been shown to exist in altered conformations.[61,62] When N3 of cytosine is protonated, it can no longer pair with guanosine in the normal Watson–Crick geometry. This could result in interesting consequences potentiating other pairing possibilities like $C.C^+$ base pairing[63–67] or C^+.G.C triplet formations[68] but the CD spectral characteristics of such structures are vastly different from that observed here.[69,70] Earlier protonation studies on poly(dG). Poly(dC) have suggested a partial dissociation of the duplex and the formation of poly(dC) self complex.[71,72] This was attributed to the presence of excess self complexed poly(dC) in the sample. Our studies indicate that the stability of the homopolymer duplex has been enhanced considerably on protonation ($T_m > 100$ °C), which rules out the formation of Hoogsteen base pairing. It is highly likely that this structure retains Watson–Crick base paring with different stacking arrangements from that of the B-form.

In the hetero-polymer, the long wavelength CD band of the H^L-form enhances in ellipticity on binding to berberine. In the homo-polymer, the negative band rapidly reduces in ellipticity, becomes zero and then develops into a positive band at the same wavelength maximum. Berberine did not however effect any changes to the Z-form CD of poly[d(G-C)]. The association of berberine molecules to the protonated structures generate extrinsic CD for the bound alkaloid molecules. Berberine is a polycondensate molecule with a positively charged nitrogen center in the C-ring (Fig. 2) and is devoid of any chiral center and thus has no intrinsic optical activity. Berberine also has no titratable groups and its structure is invariant in the pH range 1–13 as observed from absorbance and fluorescence spectra.[73] Thus, the differential effects of berberine binding to B and H^L-forms as manifested by extrinsic CD arises clearly from the conformational changes associated with the protonation of DNA and it appears that the disposition of the bound berberine molecules in the protonated and B-from structures are quite different. Detailed comparative studies of the extrinsic CD generated in the polymers suggest that in the hetero-polymer, the binding to the protonated form generate extrinsic CD of opposite sign and magnitude compared to the B-form. With the B-form structure of hetero-polymer, the extrinsic CD shows a positive band in the 420 nm region, while the same is negative with the H^L-form structure. A strong positive extrinsic CD band is generated in the 350 nm region in the B-form homo-polymer while a negative band is developed in this region for the protonated structure. It is also significant to observe that the B-form homo-polymer berberine association has a broad positive CD band in the 420 nm region while the same is absent with the protonated form. These extrinsic CD results strongly suggest that the disposition of the bound alkaloid to these protonated structures is clearly quite different from their corresponding B-form counter parts. Comparative binding data of the alkaloid to the two forms (Table 1) reveals a different picture. With both the B- and protonated forms, the alkaloid binding is non-cooperative. Although weak, the hypochromic and bathochromic effects seen with the protonated structures indicate association of the alkaloid to these structures. The binding affinity of the alkaloid has been higher with the B-form structures compared to the protonated structures and varied in the order B-form of poly[d(G-C)] > B-form of poly(dG).poly(dC) > protonated form of poly (dG).poly(dC) > H^L-form of poly[d(G-C)] (Table 1). Thus, the berberine is partially intercalating in to the protonated conformations, it is likely that a more effective coupling of its transition moments with that of the base pairs is manifested by the emergence of large

extrinsic CD patterns of opposite sign. It was suggested earlier that any drug in an intercalation site could be positioned with the long axis of the chromophore oriented parallel (classical model) or perpendicular (threading model) to the base pair long axis.[74,75] These orientations would result in the generation of extrinsic CD with opposite signs. Alternately, the two different binding modes for the alkaloid could also arise from two different orientations of the base pair; the later being detected through the berberine binding. More classical intercalators like ethidium and sanguinarine convert the protonated structure back to the B-form.[27] Our argument that berberine binding to the protonated structures represent two different orientations of the alkaloid in the helical organization of B- and protonated forms is also strengthened from the fluorescence results. While sanguinarine and ethidium presents no major variations between B-form and the protonated forms,[27] the presence of protonated forms show several fold enhancement in the intrinsic fluorescence of berberine. Almost a 20-fold enhancement in the fluorescence intensity between the B- form and the protonated form strongly reiterates the conformational differentiation of the B- and protonated forms of DNA by the alkaloid.

pH dependent conformations of DNA has gained considerable significance recently.[10–15] Studies on pH regulated gene expression and other environmental influence on DNA topology and subsequent effect on selected gene expression have been of great significance[16] as they involve a great deal of DNA structure–function relation. In this context, it is important to understand the pH induced DNA polymorphism and their binding to small molecules.

In conclusion, the emergence of the extrinsic CD bands of opposite signs with the B- and H^L-form for the bound achiral berberine molecules undoubtedly reflects two different binding geometry for berberine in these structures. This is not exhibited by other classical intercalators and is most likely due to the ability of berberine to differentially bind and discriminate the H^L- and B-conformations from its binding geometry that may essentially lead to a better understanding of the diverse biological properties of the alkaloid. In other words, the observed differential binding of berberine to B-DNA, H^L-DNA and protonated conformations may convey some specific meaning for its regulatory role in vitro and also potentiate its use as a probe for DNA conformations.

Materials and Methods

Poly(dG-dC).poly(dG-dC) [hereafter poly[d(G-C)] and poly(dG).Poly(dC) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Concentrations of the polymers were determined using molar extinction coefficients, ε (M^{-1} cm^{-1}) described earlier.[76] Berberine chloride (Sigma) was checked for purity by thin layer chromatography and melting point determinations and was used as such. A molar extinction coefficient of 22,500 M^{-1} cm^{-1} at 344 nm at pH 7.0 was used for determining its concentration. Beers law was obeyed in the range of concentrations used here. Hexaamine cobalt chloride was a product of Aldrich Chemical Co. USA. Studies were carried out in Citrate-Phosphate (CP) buffer containing 5 mM of Na_2HPO_4 as described previously.[26] The pH was adjusted in the range 2.5–7.0 by the addition of citric acid. This buffer provides constant [Na^+] of 10 mM. Deionized and double distilled water and analytical grade reagents were used throughout.

Formation of left-handed Z-, left-handed H^L and protonated structures

Z-Form structure of poly[d(G-C)] was prepared by adding B-form polymer to buffer containing 40 μM $[Co(NH_3)6]Cl_3$ kept stirred as described.[26,27] An equilibrium time of 40 min was allowed after initiating the B to Z conformational transition. The formation of the Z-structure was confirmed from UV and CD spectral measurements before and after the transition and also from the ratio of the absorbance at 260–295 nm.

The B to H^L transition was initiated by slowly adding the B-form polymer stock solution to 10 mM CP buffer, pH 3.40 kept stirred and maintained at 10 ± 1 °C as described earlier.[26,27] Hypochromicity change of 23–25% at 255 nm was generally achieved during the B to H^L transition.

Absorbance spectral studies

Absorption spectral measurements were performed on a Shimadzu UV260 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 10 ± 1 °C in thermostatically controlled cell holder as described previously.[76] Spectrophotometric titration of alkaloid–DNA complexation was performed keeping a fixed concentration of the alkaloid with varying concentrations of DNA in 1 cm path length cells to give various P/D ratios as described earlier.[77,78]

Evaluation of binding parameters

From spectrophotometric titration data, the concentration of free and bound alkaloid was estimated for each P/D, and these binding data were cast into Scatchard plots of r/C_f versus r where r is the number of moles of alkaloid bound per mole of nucleotide and C_f is the concentration of the free alkaloid. Non-linear binding isotherms observed were fitted to a theoretical curve drawn according to the excluded site model[56] for non-linear non-cooperative ligand binding phenomena using the following equation

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

where K' is the intrinsic binding constant to an isolated site, and n is the number of nucleotides occluded by the binding of a single ligand molecule. Binding data were further analyzed using the software program SCATPLOT

version 1.2[57] that works on an algorithm that determines the best fit parameters to eq. 1 as described earlier.[70]

Circular dichroism

Circular dichroic (CD) spectral measurements were performed on a Jasco J720 unit (Japan Spectroscopic Co., Ltd. Japan) equipped with a temperature controller and thermal programmer (model PTC 343) interfaced to a Compaq PC 486 in rectangular quartz cells of 1 cm path length at 10 ± 1 °C as described earlier.[36,70] Each spectrum was averaged from five successive accumulations and was base-line corrected and smoothed within the permissible limits using the software supplied by Jasco. The ellipticity values are expressed in terms of either per nucleotide (200–300 nm region) or per bound alkaloid (300–500 nm region).

Fluorescence spectroscopy

Steady state fluorescence measurements were performed on a Hitachi F-4010 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) to which was attached an Eyela Unicool UC-55 (Tokyo Rikakiki Co. Ltd., Tokyo, Japan) temperature controller. Measurements were made at 10 ± 1 °C in fluorescence free quarts cells of 1 cm path length as described earlier.[75] Uncorrected fluorescence spectra are reported.

Thermal denaturation measurements

Thermal denaturation measurements were performed on the Shimadzu UV 260 unit equipped with a temperature programmer (KPC-5) and controller (SPR-5) in stoppered matched quartz cells of 1 cm path length monitoring absorbance change at the absorption maximum of the polymers as described previously.[70]

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