Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 13 (2005) 4851–4863

Protonated structures of naturally occurring deoxyribonucleic acids and their interaction with berberine

Kakali Bhadra, Gopinatha Suresh Kumar,* Suman Das,† Md. Maidul Islam and Motilal Maiti

Biophysical Chemistry Laboratory, Indian Institute of Chemical Biology, Kolkata 700 032, India

Received 7 April 2005; revised 6 May 2005; accepted 6 May 2005
Available online 8 June 2005

Abstract—Protonation-induced conformational changes in natural DNAs of diverse base composition under the influence of low pH, low temperature, and low ionic strength have been studied using various spectroscopic techniques. At pH 3.40, 10 mM [Na^+], and at 5 °C, all natural DNAs irrespective of base composition adopted an unusual and stable conformation remarkably different from the canonical B-form conformation. This protonated conformation has been characterized to have unique absorption and circular dichroic spectral characteristics and exhibited cooperative thermal melting profiles with decreased thermal melting temperatures compared to their respective B-form counterparts. The nature of this protonated structure was further investigated by monitoring the interaction of the plant alkaloid, berberine that was previously shown from our laboratory to differentially bind to B-form and H^L-form of poly[d(G–C)] [*Bioorg. Med. Chem.* **2003**, *11*, 4861]. Binding of berberine to protonated conformation of natural DNAs resulted in intrinsic circular dichroic changes as well as generation of induced circular dichroic bands for the bound berberine molecule with opposite signs and magnitude compared with B-form structures. Nevertheless, the binding of the alkaloid to both the B and protonated forms was non-linear and non-cooperative as revealed from Scatchard plots derived from spectrophotometric titration data. Steady state fluorescence studies on the other hand showed remarkable increase of the rather weak intrinsic fluorescence of berberine on binding to the protonated structure compared to the B-form structure. Taken together, these results suggest that berberine can detect the formation of significant population of H^L-form structures under the influence of protonation irrespective of heterogeneous base compositions in natural DNAs.

1. Introduction

DNA is a structurally polymorphic macromolecule that can adopt surprising range of structures in vitro.[1,2] The right-handed antiparallel double helical structure designated as the B-form, one of the icons of the last century, can exist in a number of other conformations, viz., A- and C-forms, being widely known. The right handed B-form conformation also gets converted to the exotic left- handed Z-form in alternating guanine–cytosine polynucleotides under a variety of solution conditions, essentially retaining the Watson–Crick base pairing scheme structurally.[3] Considerable effort has been expended to unearth the biological significance of these structures.[4] Nevertheless, a detailed understanding of the physiological consequences of these conformations in vivo has been still elusive. Protonation induced structures in DNA have been very significant as pH is an important and sensitive factor in all biological process. More importantly, protonation of bases is one of the principal initiators of depurination and hence mutation. There have been several reports on the protonated structures of natural and synthetic DNAs.[5–13] In natural DNAs, protonation has earlier been suggested to induce some non-specific conformational changes that eventually lead to acid denaturation.[6] Gushlbauer and Courtois,[5] who proposed the protonation of the N7 of guanines at first in DNA, performed one of the earliest spectroscopic studies in this direction. In contrast, a subsequent Raman spectroscopic study on calf thymus DNA assigned the first protonation sites to be adenines followed by cytosines leading to the formation of a C-type conformation.[8] While supporting and contradicting reports to this model are available,[14] later studies on oligonucleotides using NMR proposed the first

Keywords: Protonated DNA; H^L-form; Berberine; Alkaloid-protonated DNA interaction; Spectroscopic study.
* Corresponding author. Tel.: +91 33 2473 4049; fax : +91 33 2473 5197/2472 3967; e-mail: gskumar@iicb.res.in
† Present address: Department of Chemistry, Raghunathpur College, Raghunathpur, Purulia 723 133, West Bengal, India.

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.05.010

protonation sites in DNA to be N3 of cytosines resulting in Hoogsteen base pairing.[15,16] Base protonation has been shown to promote the formation of triple helices,[17] H-form DNA,[18] parallel stranded structures,[19,20] and facilitate B–Z interconversion[21] in synthetic alternating guanine–cytosine polynucleotides. Our laboratory has been investigating protonation-induced conformational changes in DNAs and polynucleotides employing diverse spectroscopic tools.[8–11,22,23] From extensive spectroscopic and thermal denaturation studies, we had advanced that protonation under controlled ionic strengths and temperatures led to the formation of a unique and novel structure in alternating guanine–cytosine polynucleotides designated as H^L-form[23] while protonated form of homopolymer could not reveal any such alteration. This conformation has been characterized to be left-handed with Hoogsteen base pairing (Fig. 1). The formation of H^L-form structure was subsequently confirmed by high resolution Raman and FTIR studies.[12,24] We further employed the differential binding of the plant alkaloid berberine to the B- and H^L-form structures as a sensitive probe to differentiate the handedness of the H^L-form and B-form structures.[23] Berberine (Fig. 2) is one of the most widely distributed plant alkaloid belonging to the structural class of protoberberines and having extensive biological activities.[25–28] Briefly, berberine has strong antimutagenic and antimalarial properties and is cytotoxic toward several tumor cells.[29] Recently, the duel topoisomerase I and II poisoning activities of berberine have been revealed.[30–33] Studies also indicated that berberine induced apoptosis in human leukemia HL-60 cells.[34] Very recently the alkaloid has been implicated to have novel cholesterol lowering properties.[35] DNA-binding property of this alkaloid has been studied extensively in our and other laboratories.[36–40] A partial intercalative mode of interaction and an adenosine–thymidine (AT) base pair specificity were established from our studies[38,39] that were subsequently substantiated from NMR studies.[40,41] 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 isoquinoline moiety of berberine remained intercalated.[30,31]

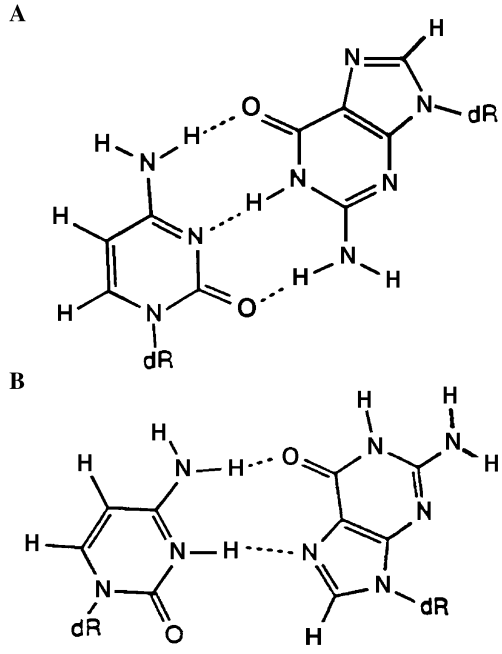

Figure 1. Structure of (A) Watson–Crick pairing and (B) Hoogsteen pairing scheme in the GC base pair.

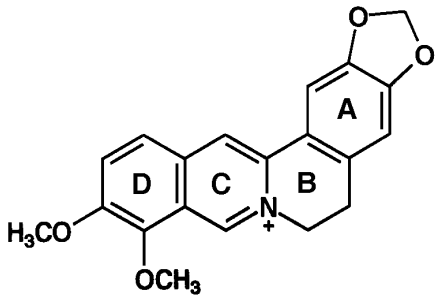

Figure 2. Chemical structure of berberine.

The molecular architecture of the H^L-form structure appears to be unique, stable, and distinctly different from the right-handed B-form and left-handed Z-form structures as revealed from our studies.[23] It has thus become pertinent to investigate whether heterogeneous sequences in natural DNAs have the potential to adopt H^L-conformations under the influence of low pH. In this communication, we have investigated the nature of protonation-induced conformations in different natural DNAs having diverse base composition using various spectroscopic tools and also used the binding of berberine to these conformations as a probe to detect the presence of H^L-form in natural DNAs.

2. Results

2.1. Circular dichroic and UV spectral changes of natural DNAs under the influence of pH

At pH 7.0, all the natural DNAs investigated here exhibited conservative circular dichroic spectra, characterized by a positive CD band around 275 nm followed by a negative band at 248 nm. On decreasing the pH, the conservative CD spectrum underwent remarkable changes. The 275 nm band exhibited hypochromic and bathochromic effects; concomitantly the negative band ellipticity also decreased. At ~pH 3.67, the ellipticity of the positive and negative bands is considerably reduced and below this pH, a dramatic change in spectral feature occurs, resulting in the formation of a spectrum with two positive bands, one centered around 253 nm and the other around 290 nm. The gross features of this spectrum do not undergo much change in the pH range 3.40–3.10. The pH dependent CD spectral changes in CP, CT, EC, and ML DNAs are presented in Figure 3.

The absorption spectral changes associated with the above observed CD changes were followed in all the four natural DNAs. As the pH was lowered, there was hypochromic effect in the 260 nm absorption band. The gross spectral changes under the influence of pH (not shown)

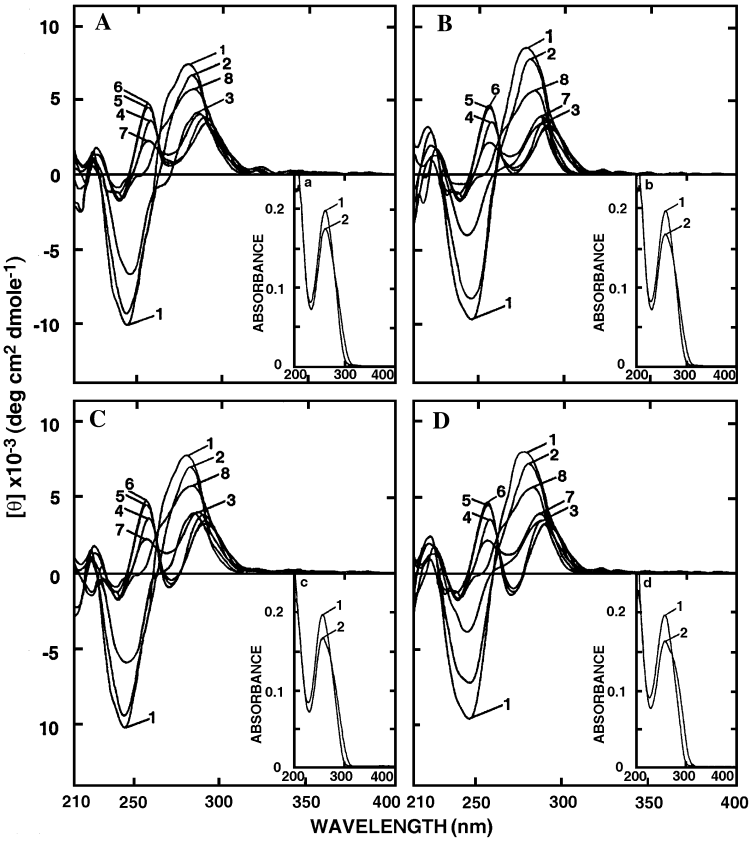

Figure 3. Influence of pH on the CD spectra of (A) CP, (B) CT, (C) EC, (D) ML DNA (64 μM in each case) with citrate–phosphate buffer at 5 ± 0.5 °C. Curves (1–8) in each case denote pH values of 7.00, 5.09, 4.21, 3.65, 3.40, 3.10, 3.01, and 2.75, respectively. Inset: UV spectra of the DNAs at pH 7.0 (curve 1) and pH 3.4 (curve 2), respectively.

were almost similar in all the DNAs, but were more pronounced in the ML DNA. At pH 3.4, a diminished UV absorption band characterizes the absorption spectra with a small hump in the 280–290 nm region (inset, Fig. 3). Further, the hypochromic effect was clearly more pronounced in the ML DNA compared to the CP DNA. The hypochromic changes observed for different DNAs being 12% for CP, 15–16% for CT and EC, and 18% for ML DNA, respectively.

2.2. Thermal denaturation

All the B-form DNAs exhibited cooperative and sharp melting transitions with 37–40% hyperchromicity changes. The melting profiles of the protonated structure at pH 3.40 also showed similar cooperative and sharp transition but the thermal melting temperature (T_m) values were remarkably lower with respect to their B-form counterparts. Representative melting profiles of the B-form and the protonated form of CT DNA are presented in Figure 4. Melting temperatures of the B-form and the protonated form of CT DNA were found to be 65 and 17 °C, respectively.

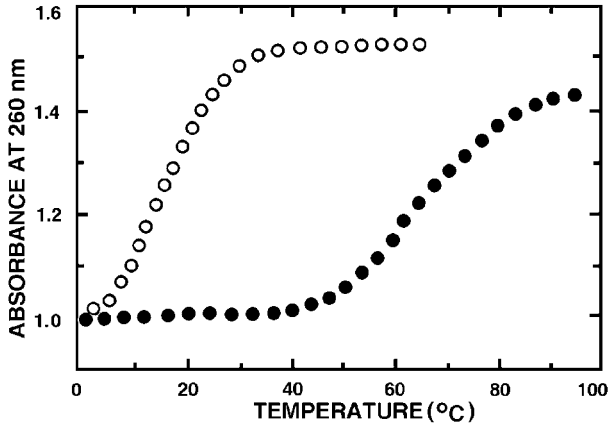

Figure 4. Representative thermal melting profiles of B-form CT DNA at pH 7.00 (●-●) and protonated form at pH 3.40 (○-○) in citrate–phosphate buffer of 10 mM [Na^+].

2.3. CD studies on the binding of berberine to the B-form and protonated form of DNA

In Figure 5, the intrinsic CD spectral changes of the B-form of all the four DNAs on interaction with berberine are presented. The characteristic B-form spectra of all the DNAs undergo changes in the presence of berberine, manifested by increase in the ellipticity of the positive band with increasing concentration of the alkaloid. It can be observed that the ellipticity of the negative band was perturbed only marginally on interaction with berberine. Similar changes were observed in all the four natural DNAs, but the extent of change was apparently more in CP DNA and varied in the order CP DNA > CT

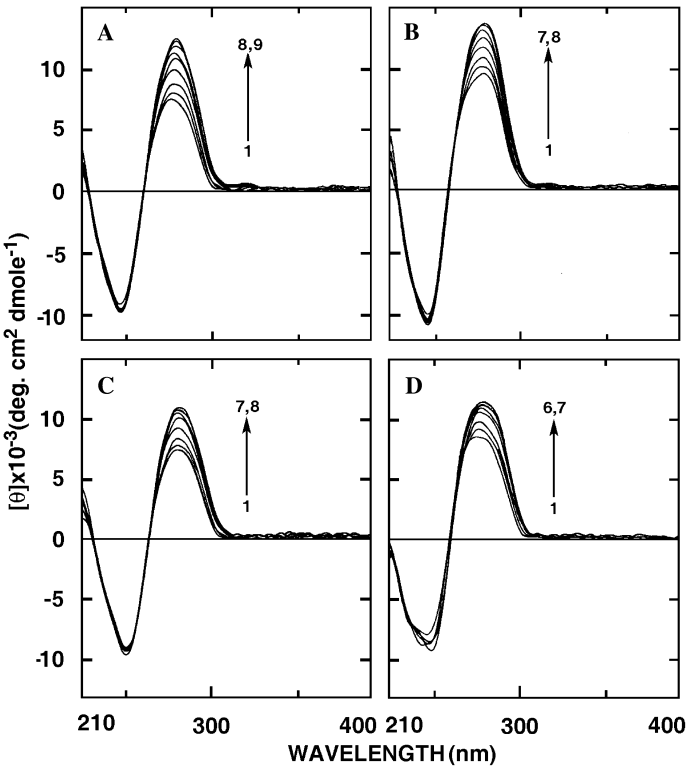

Figure 5. Representative CD spectra resulting from the interaction of berberine with the B-form DNA structure at pH 7.00 in citrate–phosphate buffer. (A) Curves (1–9) denote CP DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.8, 25.6, 32.0, 38.4, and 44.8 μM of berberine; (B) curves (1–8) denote CT DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.8, 25.6, 32.0, and 38.4 μM of berberine; (C) curves (1–8) denote EC DNA (64 μM) treated with 0, 3.15, 6.3, 12.6, 18.9, 25.2, 31.5 and 37.8 μm of berberine; (D) curves (1–7) denote ML DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.2, 25.6, and 32.0 μM of berberine, respectively.

DNA> EC DNA> ML DNA in the B-form structure at pH 7.0. It is significant to observe that apparently there was no sign of development of extrinsic CD in any of the B-form systems studied.

In Figure 6, the CD changes on the interaction of berberine with the protonated structure of all the four natural DNAs at pH 3.40 are presented. In the presence of berberine, both the two positive bands of the CD spectrum of each DNA were perturbed resulting in gradual increase in their intensity. Concomitant with the enhancement in the intrinsic CD bands, there was the appearance of a broad extrinsic positive CD band in the region 320–400 nm the ellipticity of which increased with increasing concentration of the alkaloid. The ellipticity changes of both the intrinsic and extrinsic CD bands were more pronounced in ML DNA compared to CP DNA. At saturation, the extent of molar ellipticity changes varied in the order ML DNA>EC DNA>CT DNA>CP DNA.

2.4. Extrinsic CD studies of berberine with B-form and protonated form of DNA

To gain more insight into the binding of berberine to the B-form and protonated form of DNA, extrinsic CD measurements were performed in the wavelength region 300–500 nm keeping a fixed concentration of the alkaloid (50 μM) and varying the concentration of DNA. In Figure 7, the extrinsic CD spectral changes observed on the binding of berberine to four natural DNAs are presented. Initially at low levels of binding, that is, at a *P*/*D* (nucleotide phosphate/drug molar ratio) ~10, two distinctly separated bands were observed, one of which being intense and broad with positive ellipticity with wavelength maximum in the 430–450 nm region and the other being negative in the 350 nm region. On decreasing the concentration of the DNA, changes were more pronounced in the negative band that decreased in ellipticity, became zero in magnitude, and crossed over to the positive side and became a more intense band with maximum around 355 nm. Concomitant with this, the intensity of the 440 nm band decreased. Similar extrinsic CD spectral features were observed in all the four DNAs studied. It is pertinent to observe that the molar ellipticity for the CP DNA–berberine system was the maximum and the least changes were manifested in the ML DNA.

The extrinsic CD changes on the interaction of berberine with the protonated form of four natural DNAs were similarly studied. The data are presented in Figure 8. It can be observed that binding to the protonated structure produces a negative band around 450 nm. This is exactly opposite to that which was observed with B-form structure. As the *P*/*D* decreased, the intensity of both the bands decreased. It can also be seen that the ellipticity value at any fixed *P*/*D* was larger with

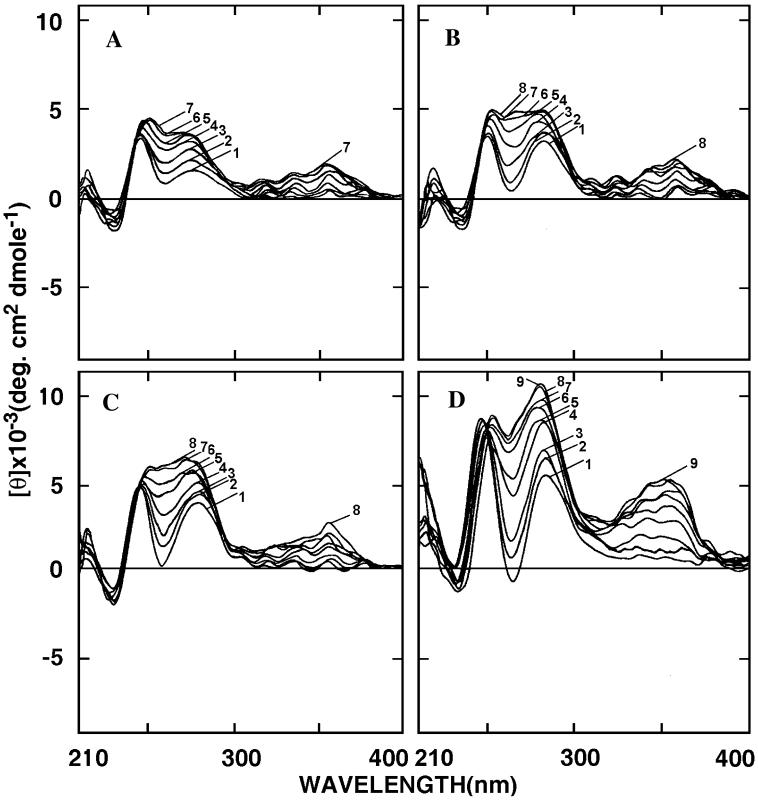

Figure 6. Representative CD spectra resulting from the interaction of berberine with the protonated form DNA structure at pH 3.40 at 5 ± 0.5 °C in citrate–phosphate buffer. (A) Curves (1–7) denote CP DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.8, 25.6, and 32.0 μM of berberine; (B) curves (1–8) denote CT DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.8, 25.6, 32.0, and 38.4 μM of berberine; (C) curves (1–8) denote EC DNA (64 μm) treated with 0, 3.15, 6.3, 12.6, 18.9, 25.2, 31.5, and 37.8 μM of berberine; and (D) curves (1–9) denote ML DNA (64 μM) treated with 0, 3.2, 6.4, 12.8, 19.2, 25.6, 32.0, 38.4 and 44.8 μM of berberine, respectively.

the GC rich ML DNA and least with AT rich CP DNA. Figure 9 shows the variation of the molar ellipticity of the 440 nm band with the GC content at a fixed *P*/*D* of 10, which followed a linear relationship in both B and the protonated form. In B-form DNA, positive molar ellipticity decreased with increasing base composition while in protonated form the negative molar ellipticity increased with increasing base composition.

2.5. Absorption spectral study

To understand the nature of the conformational changes observed in CD on binding of berberine, spectrophotometric binding studies of the alkaloid with the B-form and protonated form were performed under appropriate conditions. The effect of progressively increasing the concentration of B-form and protonated form of DNAs on the absorption spectrum of berberine is presented in Figures 10 and 11. Binding of the alkaloid results in typical hypochromic and bathochromic effects in the visible absorption spectra of berberine in both cases. The characteristic and sharp isosbestic points at 360, 380, and 440 nm indicated clearly the equilibrium in the binding phenomenon in all the cases. The results of the absorption titration were expressed in the form of Scatchard plots (inset of Figs. 10 and 11) that were analyzed according to an excluded-site model[42] using the SCATPLOT program[43] for non-cooperative binding phenomenon to fit our experimental data. We have observed that such a model adequately fits our data within the region of Scatchard plot corresponding to the range of 30% (lower) and 90% (upper) of each B-form and protonated form structure. The binding isotherm of each system is illustrated in the inset of Figures 10 and 11. The binding affinity of berberine to all the DNAs under B-form and protonated conformation is presented in Table 1. The data clearly reveal that the alkaloid has in general higher binding affinity for each of the B-form DNAs compared to the respective protonated forms. With the B-form structure, the affinity is higher with the CP DNA, while with the protonated structure, the affinity is higher with the ML DNA.

2.6. Thermal melting study of alkaloid–DNA complexation

Stabilization of B-form and protonated form structures was monitored in the presence of berberine. In the presence of the alkaloid at a fixed drug/nucleotide phosphate molar ratio (*D*/*P*) of 0.6, stabilization of B-form and protonated structures of all DNAs was observed without any significant change in the cooperativity of the transition. A maximum stabilization of about 5 °C with the B-form structure was observed in the CP DNA while the least stabilization was in the ML DNA and the stability varied in the order CP > CT > EC > ML DNA. On

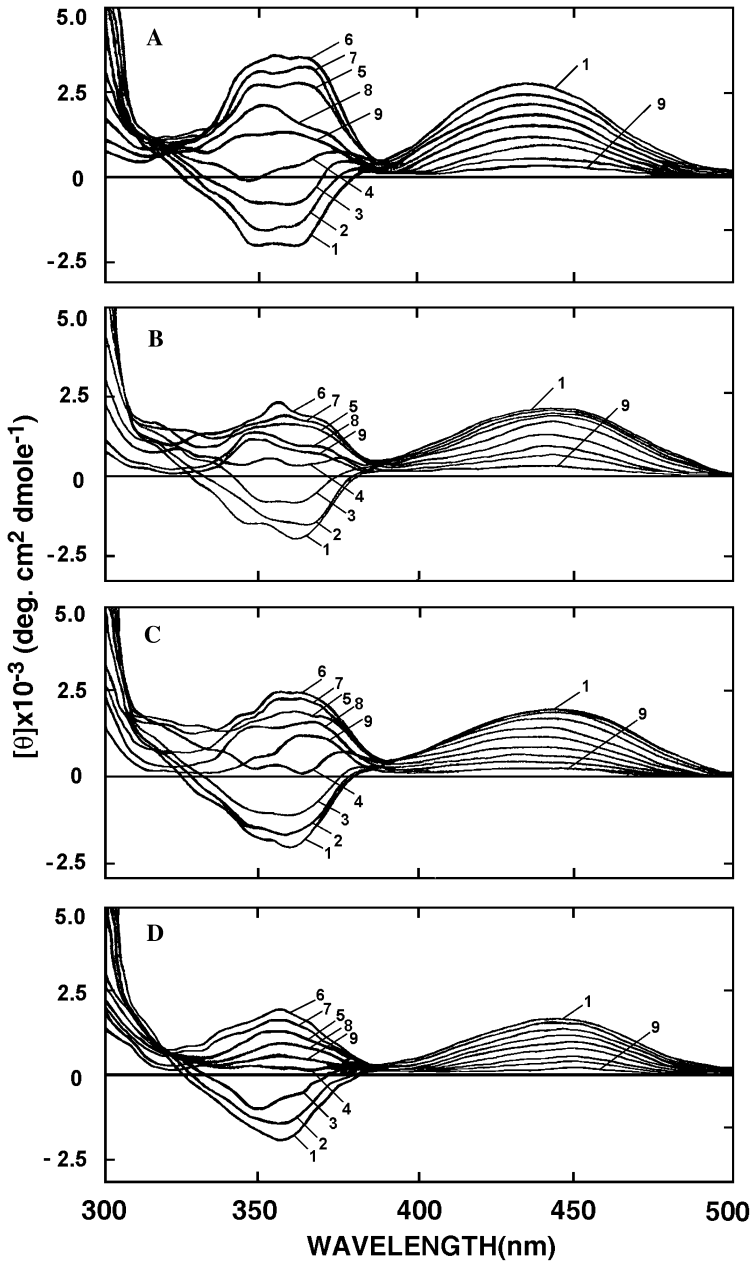

Figure 7. Extrinsic CD spectra of berberine (50 μM), treated with B-form of (A) CP, (B) CT, (C) EC, and (D) ML DNA of concentrations of 500, 400, 300, 200, 125, 75, 50, 25, and 17.5 μM as shown by curves (1–9) in each case.

the other hand, with the protonated structure a maximum stabilization of about 5 °C was obtained with the GC-rich ML DNA while least stabilization was with the CP DNA. The quantitative data on the thermal melting temperatures are depicted in Table 1.

2.7. Fluorescence spectral study

Berberine has a weak intrinsic fluorescence with a maximum around 530 nm in the range of 450–650 nm when excited at 350 nm. However, binding to DNA remarkably enhances the fluorescence intensity. In Figures 12 and 13, comparative fluorescence spectral data on the interaction of berberine with B and protonated forms of all the natural DNAs are presented. The protonated structures enhanced the fluorescence of berberine several folds (Fig. 13) compared to the B-form structure (Fig. 12) suggesting differences in the nature of the orientation or environment of the alkaloid on these structures.

2.8. pH dependent fluorescence and extrinsic CD study of the complex

To understand the effect of pH on the nature of the protonation-induced fluorescence changes, the fluorescence of berberine–CT DNA complex at a fixed *P*/*D* of 150 was studied at different pHs in the range 2.75–7.0. The result of such a study is presented in Figure 14A. It can be seen that the fluorescence intensity of the complex dramatically increased as the pH was decreased,

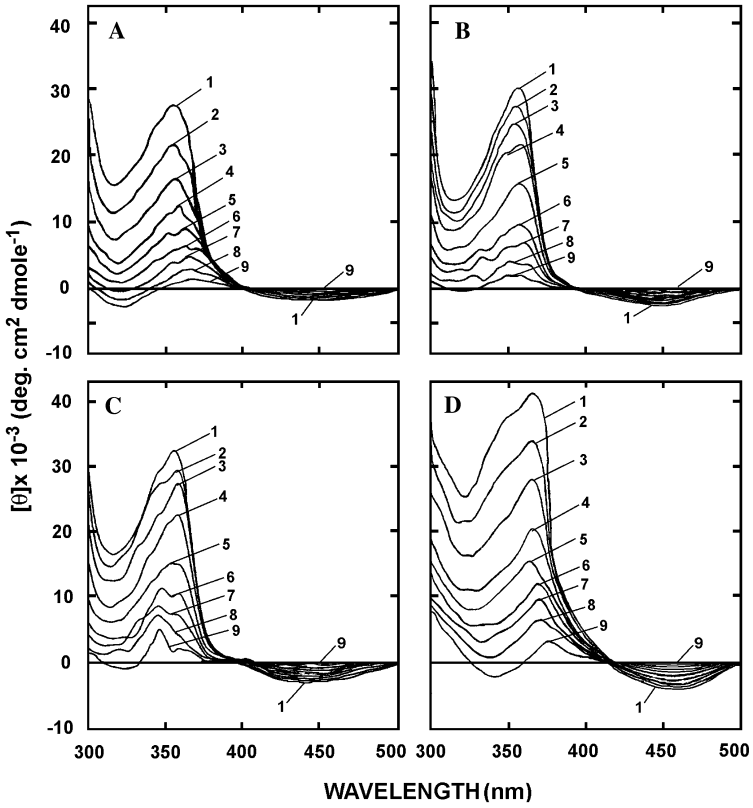

Figure 8. Extrinsic CD spectra of berberine (50 μM) treated with protonated forms of (A) CP, (B) CT, (C) EC, (D) ML DNAs of concentrations 500, 400, 300, 200, 125, 75, 50, 25, and 17.5 μM each, respectively, at 5 ± 0.5 °C as shown by curves (1–9) in each case.

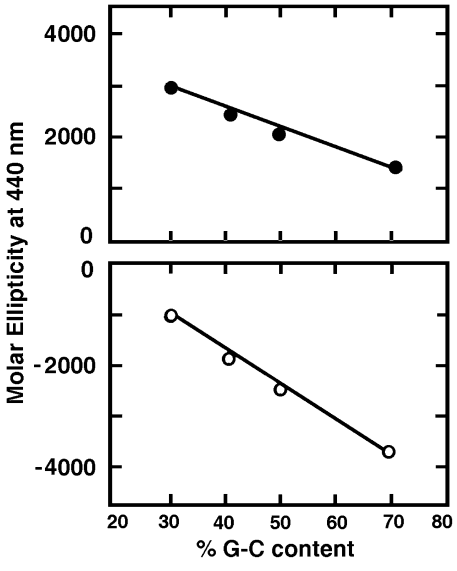

Figure 9. A plot of variation of extrinsic molar ellipticity at 440 nm of berberine–DNA complex at $P/D = 10$ against GC content of DNA. Symbol (●-●) for B-form berberine complex and (○-○) for the protonated form berberine complex.

and was maximum around a pH of 3.4, thereafter which, the fluorescence intensity dropped.

A similar study on the pH dependence of the extrinsic circular dichroism of the complex was also performed; the result is depicted in Figure 14B. Here again, the molar ellipticity of the extrinsic CD band at 355 nm enhanced as the pH was decreased, reached a maximum value at around pH 3.4, and thereafter decreased sharply, identical to the fluorescence data.

3. Discussion

In this study, we have explored the formation of H^L-structures in natural DNAs under the influence of protonation. It was observed that under the influence of pH all natural DNAs underwent systematic conformational transitions, eventually leading to a new and unique structure that remained invariant in the pH range 3.40–3.10. We will refer to this structure at pH 3.4 as the protonated structure in our subsequent discussion that is subjected to further investigation. The circular dichroic spectrum of this structure in each DNA was characterized to be non-conservative having two positive bands, one in the 290 nm and the other in the 253 nm region. This conformation appears to be significantly different from the so far known structures of natural DNAs like the A-form, the C-form, etc. Under identical conditions, our earlier study has proved that alternating guanine–cytosine polynucleotide underwent a conformational isomerization to the H^L-conformation that resulted in a characteristic CD pattern with a negative band in the 290 nm.[23] It was also observed that the right-handed B-form, the left-handed Z-form and the left-handed Hoogsteen base paired H^L-form structures

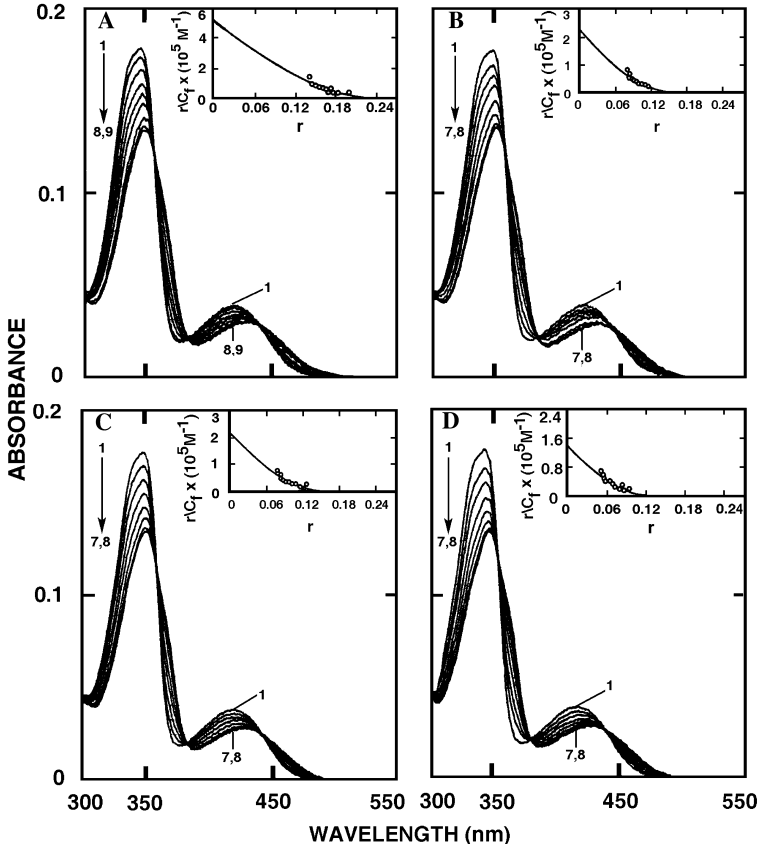

Figure 10. Representative absorption spectral changes in the presence of B-form DNAs. (A) Curves (1–9) denote absorption spectrum of berberine (8.00 μM) treated with 0, 7.40, 14.80, 25.60, 36.80, 47.92, 55.28, 62.40, and 72.00 μM of CP DNA; (B) curves (1–8) denote absorption spectrum of berberine (8.00 μM) treated with 0, 7.28, 12.26, 40.80, 63.04, 89.28, 117.68, and 125.0 μM of CT DNA; (C) curves (1–8) denote absorption spectrum of berberine (8.00 μM) treated with 0, 8.75, 20.07, 34.33, 51.38, 90.84, 120.0, and 130 μM of EC DNA; and (D) curves (1–8) denote absorption spectrum of berberine (8.00 μM) treated with 0, 12.90, 41.86, 61.11, 80.31, 137.64, 169.68, and 175.0 μM of ML DNA, respectively, in citrate–phosphate buffer, 10 mM [Na^+], pH 7.0 at 5 ± 0.5 °C. Inset: representative Scatchard plots for the binding in each case. 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. This model adequately fits the data within the regions of the Scatchard plot ranging from 30% (lower limit) to 90% (upper limit). Values of K and n are presented in Table 1.

exhibited an isoelliptic point in CD.[22] Although such negative CD band in the 290–300 nm characteristic of typical left-handed conformation was not achievable in natural DNAs, probably due to the likely heterogeneous distribution of base sequences, the trend in the circular dichroic changes appears to lead to similar conformational transitions in natural DNAs. It is also pertinent to observe that the changes were more apparent in the GC-rich ML DNAs confirming the propensity of the guanine–cytosine base pairs to undergo transition to Hoogsteen pairing. The ultraviolet spectral changes that accompany the CD changes reflect a hypochromic effect in the 260 nm band in all the DNAs, being more in the GC-rich ML DNA and the least in the AT rich CP DNA. Further in the ML DNA, there was also the formation of a small hump in the 290 nm region of the UV spectrum similar to that observed in the alternating G–C sequences.[22] The double helical nature of the protonated structure in each DNA was ascertained by characterizing through thermal melting profiles. In all the DNAs, cooperative thermal melting profiles were seen in both the B and the protonated structures without much variation in the cooperativity or hyperchromicity of the transitions, clearly underscoring that the structure that is under investigation is a native double helical structure.

More information about the nature of the protonated state was obtained from studies with the interaction of the isoquinoline alkaloid berberine. Binding of berberine within the chiral environment of DNA can lead to induced optical activity of the bound species that is dependent on the nature and handedness of the host molecules. Berberine has indeed been previously shown to differentially bind to the right-handed B- and left-handed H^L-forms of the alternating GC polynucleotide generating CD spectra with opposite signs and magnitude.[23] Binding to the B-form of the natural DNAs resulted in changes in CD that is characteristic of intercalating compounds resulting in typical enhancement of the long wavelength CD band. In sharp contrast, the changes with the protonated structure was significantly more pronounced and resulted in changes of higher magnitude. Additionally, the association of the alkaloid with the protonated forms of all the DNAs generated extrinsic circular dichroic bands with remarkably high intensities that increased as the binding in-

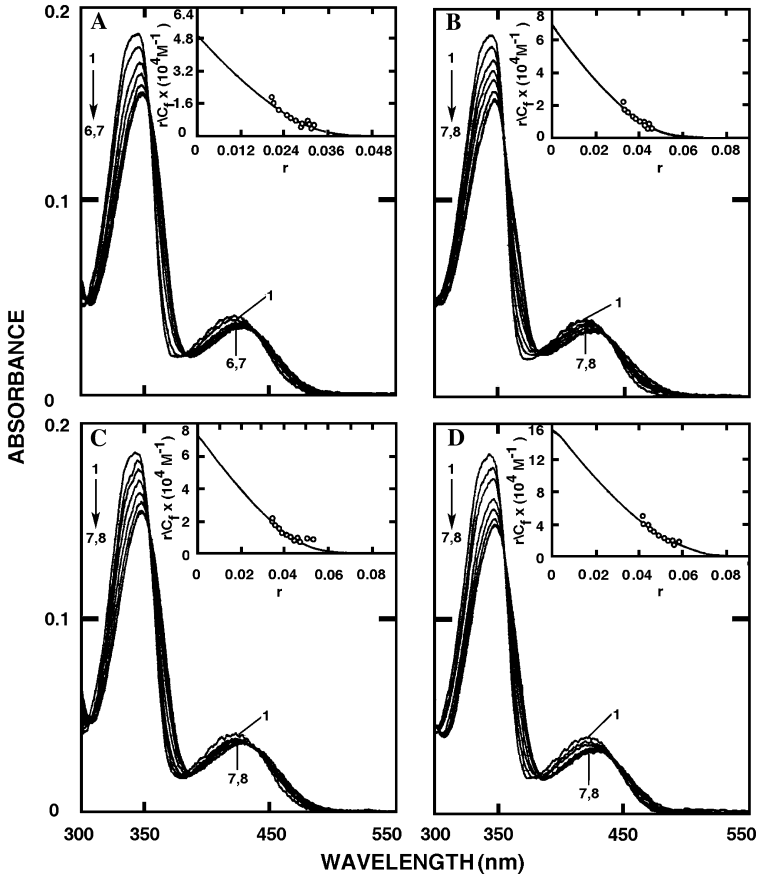

Figure 11. Representative absorption spectral changes in the presence of protonated DNAs. (A) Curves (1–7) denote absorption spectrum of berberine (8.26 μM) treated with 0, 41.33, 103.33, 247.98, 330.64, 454.63, and 550 μM of CP DNA; (B) curves (1–8) denote absorption spectrum of berberine (8.26 μM) treated with 0, 41.33, 82.66, 144.66, 206.65, 247.98, 330.64, and 420 μM CT DNA; (C) curves (1–8) denote absorption spectrum of berberine (8.26 μM) treated with 0, 41.33, 82.66, 123.99, 206.65, 268.65, 330.64, and 420.0 μM of EC DNA, and (D) curves (1–8) denote absorption spectrum of berberine (8.26 μM) treated with 0, 41.33, 82.66, 125.99, 165.32, 227.32, 289.31, and 350 μM of ML DNA, respectively, in citrate–phosphate buffer of 10 mM [Na^+] at pH 3.40 at 5 ± 0.5 °C. Inset: representative Scatchard plots for the binding in each case. 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. This model adequately fits the data within the regions of the Scatchard plot ranging from 30% (lower limit) to 90% (upper limit). Values of K and n are presented in Table 1.

Table 1. Binding parameters and ΔT_m values of the interaction of berberine with B-form and protonated form of natural DNAs[a]

DNA	GC (mol %)	B-form[b]			Protonated form[c]		
		K ($\times 10^5$ M^{-1})	n	ΔT_m[d]	K ($\times 10^4$ M^{-1})	n	ΔT_m[d]
CP	30	4.5	3.8	5.0	5.0	20.0	1.0
CT	42	2.2	6.0	3.0	7.0	14.0	3.0
EC	50	2.1	6.0	2.0	7.1	13.8	3.5
ML	72	1.35	7.0	2.0	15.0	12.0	5.0

[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 alkaloid–DNA complex − T_m of DNA]. T_m of native B-form CP, CT, EC, and ML DNAs were 51, 65, 67.5, and 77.5 °C while that of their protonated counterparts were 13,17,18, and 22.5 °C, respectively.

creased. Such induced CD bands were not seen in the intrinsic CD experiments with the B-form structures. Berberine is a polycondensate molecule with partial saturation in the ring B. But the structure is completely devoid of any chiral centers and hence does not have any CD spectrum. Hence, the generation of strong extrinsic CD in the bound berberine molecules manifests from its asymmetric organization on the protonated structure that is clearly different from that of its bound arrangement on the right-handed B-DNA structure. This in turn may also be indicative of the different orientations of the bound berberine in these two conformations.

Detailed comparative results on the extrinsic CD spectra generated in the B-form and the protonated form studied from titration experiments, keeping a constant

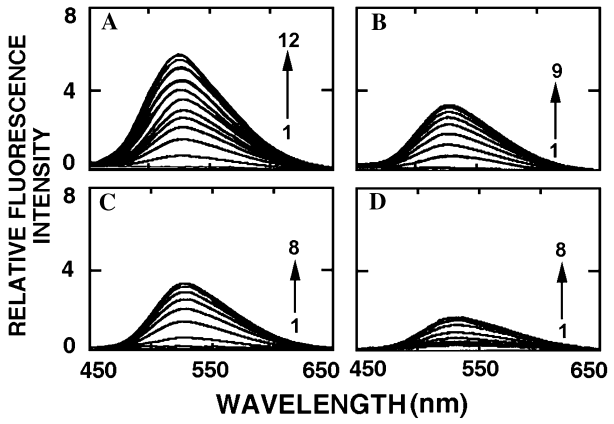

Figure 12. Representative steady state fluorescence emission spectrum of berberine (3.5 μM) treated with B-form DNAs. (A) Curves (1–12) denote 0, 5.25, 6.75, 10.25, 20.27, 33.79, 45.30, 52.36, 60.81, 70.33, 87.84, and 101.36 μM of CP DNA; (B) curves (1–9) denote 0, 7.12, 23.13, 32.45, 42.30, 52.98, 69.73, 84.62, and 100.24 μM of CT DNA; (C) curves (1–8) denote 0, 6.65, 20.13, 32.55, 41.35, 53.98, 69.40, and 84.82 μM of EC DNA; and (D) curves (1–8) denote 0, 6.65, 21.23, 33.65, 43.56, 53.26, 67.34, and 83.68 μM of ML DNA, respectively, at 5 ± 0.5 °C in citrate–phosphate buffer, pH 7.0.

concentration of the alkaloid and varying the concentration of the nucleic acid, throw light on the differential orientation and/or arrangement of berberine on the different helical organization of the DNA under these two conditions. Binding to the B-form results in extrinsic CD with a positive CD band in the 440 nm followed by a negative CD band in the 350 nm region while with the protonated form a negative band is generated in the 440 nm region followed by a remarkably large positive band around 355 nm. This observation is similar to that has been observed in the B- and H^L-forms of alternating GC polymer where berberine was shown to be differentially disposed on the right-handed Watson–Crick base paired B and the left-handed Hoogsteen base paired H^L-forms.[23] Similar observation here in B-form and protonated form undoubtedly suggests the formation of significant population of H^L-structures in the protonated state of natural DNAs. Another significant aspect that has emerged from the intrinsic and extrinsic CD data is the base pair dependence of binding of the alkaloid to these structures. With the B-form conformation, changes were more pronounced in the AT rich CP DNA while with the protonated structure, an exactly opposite trend namely the GC base pair specificity was seen. Apparently, berberine appears to be AT specific with B-form and GC specific with protonated structures. This was also substantiated from other studies as well as discussed below.

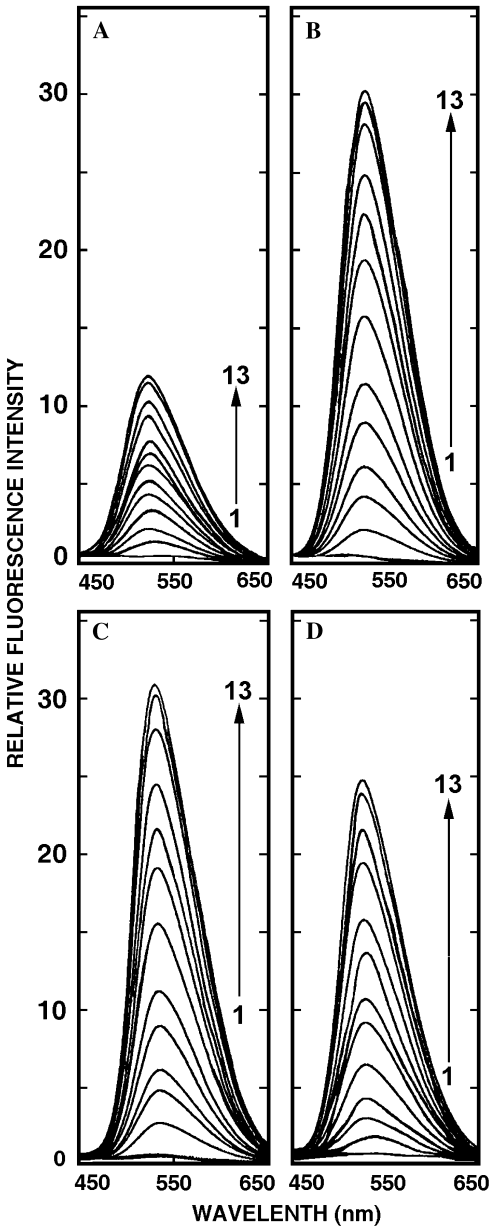

Figure 13. Representative steady state fluorescence emission spectrum of berberine (3.5 μM) treated with protonated DNAs. (A) Curves (1–13) denote 0, 7.00, 26.25, 35.00, 52.5, 70.0, 105, 140, 210, 280, 350, 437.5 and 525 μM of CP DNA; (B) curves (1–13) denote 0, 6.32, 25.32, 34.48, 50.48, 71.32, 103.56, 139.92, 220, 285, 360, 440, and 530 μM of CT DNA; (C) curves (1–13) denote 0, 6.40, 24.32, 34.90, 52.48, 71.54, 105.6, 138.92, 220, 285, 360, 441, and 532 μM of EC DNA; and (D) curves (1–13) denote 0, 6.65, 21.23, 33.65, 53.26, 67.34, 104.66, 138.92, 220, 284.32, 360, 441, and 530 μM of ML DNA, respectively, at 5 ± 0.5 °C in citrate–phosphate buffer, pH 3.40.

Comparative binding data of berberine to these structures in natural DNAs were derived from Scatchard analysis. The binding affinity of the alkaloid is higher with the B-form conformation in each case compared to the protonated conformation. With the B-form, the affinity varied in the order CP DNA > CT DNA > EC DNA > ML DNA, while with the protonated structures an exactly opposite behavior was seen. Apparently, the binding data also suggest that the affinity with the B-form structure is higher with the CP DNA, while with the protonated conformation the affinity is more towards the ML DNA. It is interesting to observe that the number of binding sites as denoted by the '*n*' value (Table 1) is lower in B-form while it is higher with the protonated form.

The results of spectroscopic, circular dichroic, and binding affinity data have provided a clear picture of the base

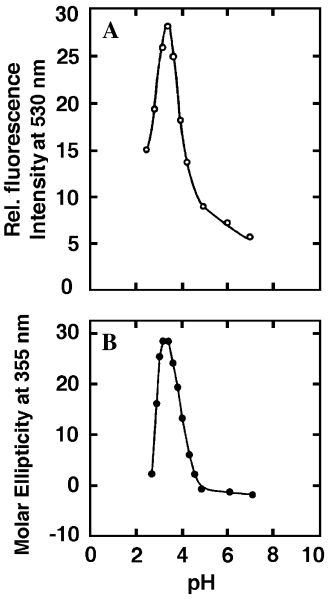

Figure 14. Influence of pH on berberine–DNA complexation obtained from (A) spectrofluorimetric study at $P/D = 150$ and (B) circular dichroic study at $P/D = 10$.

pair specificity of berberine with B-form and protonated conformation. The strong affinity of berberine to AT base pairs in B-form DNA has been well documented in the literature.[38–41] Our comparative studies with the B-form natural DNAs further confirm this. In sharp contrast, this affinity switches to GC base pairs when the conformation of DNA changes under the influence of protonation. This is revealed from the higher binding affinity, larger CD, and fluorescence changes for the ML DNA compared to CP DNA at pH 3.4. Taken together, these results confirm that berberine switches the specificity of binding from AT base pairs with B-form to GC base pairs with protonated form. To our knowledge, this is therefore the first example of an alkaloid molecule exhibiting different base specificities being controlled by the DNA conformation.

pH dependent conformations of DNA have gained considerable significance recently.[13,44–46] Studies on pH regulated gene expression and other environmental influence on DNA topology, and subsequent effect on selected gene expression has been of great significance 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. The polycyclic aromatic hydrocarbon pyrene has previously been shown to bind to DNA exhibiting pH dependent extrinsic CD spectra.[47] Recent studies on the pH dependent binding of the cancer chemopreventive agent curcumin and the alkaloid aristololactam-β-D-glucoside to natural and synthetic DNAs underscore the importance of pH dependent DNA conformations on the binding phenomenon of these molecules.[48,49] Canonical B-form duplex structure does not possess any strong protonation sites and consequently it does not pick up protons. Nevertheless, novel structures like the H^L-form involving Hoogsteen base pairing is possible for protonated dG.dC$^+$ base pairs. It was suggested by Munten and Seagers-Norton[46] that a subtle interplay between pH and ionic concentration may result in specific changes in DNA conformation. In our work, we have unequivocally observed that by controlling the ionic strength and temperature all the natural DNAs could adopt a similar duplex conformation at pH 3.4 most likely involving the protonated alternating guanine–cytosine base pairing in Hoogsteen type (H^L-form) resulting in significant left handed conformation.

It is also perhaps pertinent to comment on the mode of interaction of berberine with the protonated conformation and the B-form structure that leads to the emergence of CD signals with opposite signs and magnitude and remarkable enhancement of fluorescence of berberine. Berberine is a polycondensate molecule that can partially intercalate within the base pairs of DNA.[38–40] Induced circular dichroism arises from an effective interaction between transition moments of DNA bases and chirally arranged berberine molecules, the sign and magnitude of induced CD bands being largely dependent on the orientation of the bound molecule at the binding site.[50] In both B-form and the protonated form, we have observed strong hypochromism and bathochromism, which lend strong support to similar modes of interaction for berberine with both structures. Thus, if berberine is partially intercalating in both the structures, then, the emergence of extrinsic CD bands of opposite signs with the B-form and H^L-form for the bound achiral berberine molecules in these two conformations undoubtedly reflects two different binding geometries for berberine in these structures, which is likely due to berberine being arranged in two different assymmetric organizations, being right-handed on the B-form and left-handed on the protonated form. It is also pertinent to observe that such a differentiation is not exhibited by other classical intercalators like sanguinarine and ethidium[22] that convert the protonated form to B-form and therefore is undoubtedly due to the ability of berberine to differentially bind and discriminate the B- and protonated 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-form and protonated conformation may potentiate its use as a probe for DNA conformations, which in turn may convey some specific meaning for its regulatory role in vivo.

4. Materials and methods

Clostridium perfringens (CP) DNA (Type XII, 30% GC); Calf thymus (CT) DNA (Type I, 42% GC); *Escherichia coli* (EC) DNA (Type VII, 50% GC) and *Micrococcus lysodeikticus* (ML) DNA (Type XI, 72% GC) were obtained from Sigma (St. Louis, MO, USA). Each DNA exhibited a characteristic ultraviolet absorption spectrum with an A_{260}/A_{280} ratio between 1.88 and 1.93 and an absorption of A_{260}/A_{230} ratio between 2.12

and 2.22. DNA concentrations in terms of nucleotide phosphate were determined in buffer of pH 7.0 spectrophotometrically as described earlier[49] using molar extinction coefficients ε (M^{-1} cm^{-1}) at 260 nm for CP DNA (6300), CT DNA (6600), EC DNA (6500), and ML DNA (6900). The alkaloid, berberine chloride, was a product of Sigma and was checked for its purity by thin layer chromatography and melting point before use. Since no visible impurities were observed, no further purification was done. The solution of the alkaloid was freshly prepared each day and was kept protected in dark. An extinction coefficient ε (M^{-1} cm^{-1}) of 22,500 at 344 nm at pH 7.0 was used for determining its concentration. Beer's law was obeyed in the concentration range employed here. Citrate–phosphate (CP) buffer containing 5 mM of Na_2HPO_4 as described previously[11] was used for pH dependent studies. 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. pH measurements were made on an EC digital pH meter (Electronics Corporation of India) with an accuracy of ±0.02 units. Deionized double distilled water and analytical grade reagents were used throughout. All the buffer solutions were filtered through Millipore filters of 0.45 μm to remove any particulate matter.

4.1. Formation of protonated structures

The B-form to protonated structure conformational transition was initiated by slowly adding the DNA stock solution to 10 mM CP buffer, pH 3.4, kept stirred and maintained at 5.0 ± 0.5 °C as described earlier.[11,22,23] Generally, the structural transition from B- to protonated structure followed very fast kinetics and was found to be complete in about 1 s. In practice equilibrium of 5 min was allowed before measurements were performed. Dry nitrogen gas was purged through the sample chamber to avoid moisture condensation on the optical windows. Hypochromicity change of about 15–20% at 260 nm was generally achieved during the B-form to protonated conformational transition in each natural DNA.

4.2. Absorbance spectral studies

All UV–vis absorbance measurements were made on a Shimadzu UV260 automatic recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 5.0 ± 0.5 °C equipped with a thermostatically controlled cell holder as described previously[51] in matched 1 cm path length quartz cells. A thermoprogrammer (KPC-5) in conjunction with a temperature controller (SPR-5) was used to maintain the sample temperature. Spectrophotometric titration of alkaloid–DNA complexation was performed keeping a fixed concentration of the alkaloid and varying the concentration of DNA in 1 cm path length cells to give various P/D ratios as described earlier.[52,53]

4.3. Evaluation of binding parameters

From spectrophotometric titration data, the concentration of free and bound alkaloid was estimated for each P/D ratio. These 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[42] 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 alkaloid molecule. Binding data were further analyzed using the software program SCATPLOT[43] version 1.2 that works on an algorithm that determines the best fit parameters to Eq. 1 as described elsewhere.[54]

4.4. Circular dichroism measurements

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 was used to record circular dichroic spectra in rectangular quartz cells of 1 cm path at 5 ± 0.5 °C as described earlier.[54,55] Each spectrum was averaged from five successive accumulations with a band width of 1.0 nm and a sensitivity of 100 millidegree at a scan speed of 100 nm min^{-1} and was baseline corrected and smoothed within the permissible limits using the software supplied by Jasco. The ellipticity values are expressed in terms of either per nucleotide (200–400 nm region) or per bound alkaloid (300–500 nm region). The CD unit was routinely calibrated using solutions of d-10 ammonium camphor sulfonate.

4.5. Fluorescence spectroscopy

A Hitachi F-4010 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) with an attached Eyela Unicool UC-55 (Tokyo Rikakiki Co. Ltd., Tokyo, Japan) temperature controller was used to measure the steady state fluorescence spectra. Measurements were made at 5 ± 0.5 °C in fluorescence free quartz cells of 1 cm path length as described earlier.[54] Uncorrected fluorescence spectra are reported.

4.6. 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 260 nm as described previously.[55]

Acknowledgments

Kakali Bhadra and Md. Maidul Islam were supported by Junior Research Fellowships of the Council of Scientific and Industrial Research (CSIR) Government of

India. Suman Das was a Senior Research Fellow of UGC, Government of India.

References and notes

1. Saenger, W. *Principles of Nucleic acid Structure*; Springer: New York, 1984.
2. Belmont, P.; Constant, J-F.; Demeunynck, M. *Chem. Soc. Rev.* **2001**, *30*, 70.
3. Rich, A.; Nordheim, A.; Wang, A. H. J. *Annu. Rev. Biochem.* **1984**, *53*, 791.
4. Herbert, A.; Rich, A. *J. Biol. Chem.* **1996**, *271*, 11595.
5. Guschlbauer, W.; Courtois, Y. *FEBS Lett.* **1968**, *1*, 183.
6. Zimmer, Ch.; Luck, G.; Venner, H. *Biopolymers* **1968**, *6*, 563.
7. O' Connor, T.; Mansy, S.; Bina, M.; Mc Millin, D. R.; Bruck, M. A.; Tobias, R. S. *Biophys. Chem.* **1981**, *15*, 53.
8. Maiti, M.; Nandi, R. *Indian J. Biochem. Biophys.* **1986**, *23*, 322.
9. Maiti, M.; Nandi, R. *Indian J. Biochem. Biophys.* **1987**, *24*, 96.
10. Maiti, M.; Nandi, R. *Anal. Biochem.* **1987**, *164*, 68.
11. Kumar, G. S.; Maiti, M. *J. Biomol. Struct. Dyn.* **1994**, *12*, 183.
12. Segers-Nolten, G. M. J.; Sijtsema, N. M.; Otto, C. *Biochemistry* **1997**, *36*, 13241.
13. Airoldi, M.; Boicelli, C. A.; Cadoni, F.; Gennaro, G.; Giomini, M.; Giuliani, A. M.; Giustin, M. *Biopolymers* **2004**, *75*, 118.
14. Banville, D. L.; Marzilli, L. G.; Wilson, W. D. *Biochemistry* **1986**, *25*, 7393.
15. Topping, R. J.; Stone, M. P.; Brush, C. K.; Harris, T. M. *Biochemistry* **1988**, *27*, 7216.
16. Robinson, H.; van der Marel, G. A.; van Boom, J. H.; Wang, A. H. J. *Biochemistry* **1992**, *31*, 10510.
17. Bhaumik, S. R.; Chary, K. V. R.; Govil, G.; Liu, K.; Miles, H. T. *Nucleic Acids Res.* **1995**, *23*, 4116.
18. Frank Kaminiteski, M. D. *Nucleic Acid and Molecular Biology*; Springer: Berlin, 1990, p 1.
19. Dolinnya, N. G.; Ulku, A.; Fresco, J. R. *Nucleic Acids Res.* **1997**, *25*, 1100.
20. Singh, S.; Patel, P. K.; Hosur, R. V. *Biochemistry* **1997**, *36*, 13214.
21. Chen, F. M. *Biochemistry* **1984**, *23*, 6159.
22. Das, S.; Kumar, G. S.; Maiti, M. *Biophys. Chem.* **1999**, *76*, 199.
23. Kumar, G. S.; Das, S.; Bhadra, K.; Maiti, M. *Bioorg. Med. Chem.* **2003**, *11*, 4861.
24. Tajmir-Riahi, H. A.; Neault, J. F.; Naoui, M. *FEBS Lett.* **1995**, *370*, 105.
25. Suffness, M.; Cordell, A. G. In *The Alkaloids*; Brossi, A., Ed.; Academic: New york, 1985, p 3.
26. Creasey, W. A. *Biochem. Pharmacol.* **1979**, *28*, 1081.
27. Inbaraj, J. J.; Kukielezak, B. M.; Bilski, P.; Sandvik, S. L.; Chignell, C. F. *Chem. Res. Toxicol.* **2001**, *14*, 1529.
28. Kuo, C. L.; Chi, C-W.; Liu, T-Y. *Cancer Lett.* **2004**, *203*, 127.
29. Cernakova, M.; Kostalova, D.; Kettmann, V.; Plodova, M.; Toth, J.; Drimal, J. *BMC Complementary and Alternative Medicine* **2002**, *2*, 1.
30. Pilch, D. S.; Yu, C.; Makhey, D.; La Voic, E. J.; Srinivasan, A. R.; Olson, W. K.; Sauers, R. R.; Breslauer, K. J.; Geacintov, N. E.; Liu, L. F. *Biochemistry* **1997**, *36*, 12542.
31. Kim, S. A.; Kwon, Y.; Kim, J. H.; Muller, M. T.; Chung, I. K. *Biochemistry* **1998**, *37*, 16316.
32. Li, T. K.; Bathory, E.; La Voie, E. J.; Srinivasan, A. R.; Olson, W. K.; Sauers, R. R.; Liu, L. F.; Pilch, D. S. *Biochemistry* **2000**, *39*, 7107.
33. Krishnan, P.; Bastow, K. F. *Anti-Cancer Drug Des.* **2000**, *15*, 255.
34. Wu, H. L.; Hsu, C. Y.; Liu, W. H.; Yung, B. Y. M. *Int. J. Cancer* **1999**, *81*, 923.
35. Kong, W.; Wei, J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H.; Wang, S.; Wu, J.; Wang, Y.; Li, Z.; Liu, J.; Jiang, J-D. *Nat. Med.* **2004**, *10*, 1344.
36. Davidson, M. W.; Lopp, I.; Alexander, S.; Wilson, W. D. *Nucleic Acids Res.* **1977**, *4*, 2697.
37. Maiti, M.; Chaudhuri, K. *Indian J. Biochem. Biophys.* **1981**, *18*, 245.
38. Debnath, D.; Kumar, G. S.; Nandi, R.; Maiti, M. *Indian J. Biochem. Biophys.* **1989**, *26*, 201.
39. Debnath, D.; Kumar, G. S.; Maiti, M. *J. Biomol. Struct. Dyn.* **1991**, *9*, 61.
40. Saran, A.; Srivastava, S.; Coutinho, E.; Maiti, M. *Indian J. Biochem. Biophys.* **1995**, *32*, 74.
41. Mazzini, S.; Bellucci, M. C.; Mondelli, R. *Bioorg. Med. Chem.* **2003**, *11*, 505.
42. McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.
43. Ray, A.; Maiti, M.; Nandy, A. *Comput. Biol. Med.* **1996**, *26*, 497.
44. Muntean, C. M.; Puppels, G. J.; Greve, J.; Seagers-Nolten, G. M. J. *Biopolymers* **2002**, *67*, 282.
45. Muntean, C. M.; Puppels, G. J.; Greve, J.; Seagers-Nolten, G. M. J.; Cinta-Pinzaru, S. *J. Raman Spectrosc.* **2002**, *33*, 784.
46. Muntean, C. M.; Seagers-Nolten, G. M. J. *Biopolymers* **2003**, *72*, 225.
47. Chen, F-M. *J. Biomol. Struct. Dyn.* **1983**, *1*, 925.
48. Zsila, F.; Bikadi, Z.; Simonyi, M. *Org. Biomol. Chem.* **2004**, *2*, 2902.
49. Ray, A.; Kumar, G. S.; Das, S.; Maiti, M. *J. Biomol. Struct. Dyn.* **2003**, *21*, 141.
50. Monnot, M.; Mauffret, O.; Lescot, E.; Fermandjian, S. *Eur. J. Biochem.* **1992**, *204*, 1035.
51. Sen, A.; Ray, A.; Maiti, M. *Biophys. Chem.* **1996**, *59*, 155.
52. Maiti, M.; Nandi, R.; Chaudhuri, K. *FEBS Lett.* **1982**, *142*, 280.
53. Nandi, R.; Chakraborty, S.; Maiti, M. *Biochemistry* **1991**, *30*, 3715.
54. Ray, A.; Maiti, M. *Biochemistry* **1996**, *35*, 7394.
55. Ray, A.; Kumar, G. S.; Das, S.; Maiti, M. *Biochemistry* **1999**, *38*, 6239.