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Berberine, a strong polyriboadenylic acid binding plant alkaloid: spectroscopic, viscometric, and thermodynamic study

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Abstract—The interaction of berberine with single stranded poly(rA) structure was investigated using a combination of spectrophotometric, spectrofluorimetric, circular dichroic, viscometric, and thermodynamic studies. The interaction process was characterized by typical hypochromic and bathochromic effects in the absorption spectrum of berberine, enhancement of fluorescence intensity of berberine, increase of viscosity, and perturbation of circular dichroic spectrum of single stranded poly(rA). Scatchard plot obtained from spectrophotometric analysis showed that berberine bound strongly to single stranded poly(rA) in a non-cooperative manner. In contrast, berberine does not show any significant effect (i) in its absorbance and fluorescence spectra on binding to double stranded poly(rA), (ii) alter the circular dichroic spectrum of double stranded poly(rA), or (iii) increase of viscosity of double stranded poly(rA) indicating that it does not bind at all to double stranded poly(rA) structure. Thermodynamic parameters indicated that the binding of the alkaloid to single stranded poly(rA) is an endothermic process and entropy driven. All these findings, taken together clearly support that berberine binds strongly to single stranded poly(rA) structure by a mechanism of partial intercalation leading to its use in gene regulation in eukaryotic cells.

1. Introduction

Polyriboadenylic acid [poly(rA)] has been established to exist as a single stranded helix stabilized by pairwise stacking interaction between adjacent bases at physiological pH and temperature.[1,2] The helical conformation, however, gets gradually converted to random coil with increasing temperature. At pH $\leqslant 4.5$, poly(rA) adopts a parallel stranded double helical conformation with a single groove in which the adenine–adenine base pairs are significantly tilted from the plane perpendicular to the helix axis.[3] The molecular structure of the double helical poly(rA) has been established from X-ray crystallographic analysis and has been proved to be quite different from those of double standard RNA double helices.[3] It is known that poly(rA) structure plays an essential role in gene expression in eukaryotic cells.[4–7] Polyadenylation of primary transcripts is an essential event for the maturation of eukaryotic mRNAs and poly(rA) tail confers the necessary stability for mRNA molecules.[4] A schematic representation of poly(rA) tail in transcription

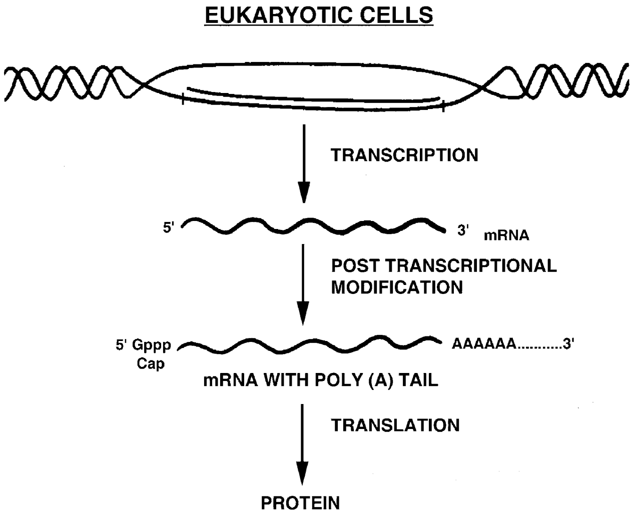

Figure 1. A schematic diagram for transcription process in eukaryotic cells.

Keywords: Berberine; Poly(A); Alkaloid–poly(A) complexation.

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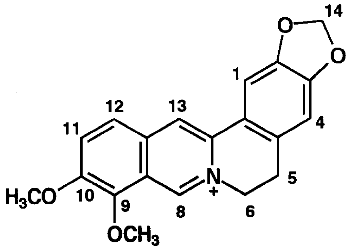

Figure 2. Chemical structure of berberine.

process in eukaryotic cells is shown in Figure 1. Polyadenylation process also plays a significant regulatory role in the production of alternative forms of proteins.[5,6] Recently, Zarudnya and Hovorun[7] proposed a possible biological role for double stranded poly(rA) structure and suggested the involvement of double stranded oligo(rA) sequence in intracellular process as termination of mRNA-poly(rA) synthesis and auto regulation of poly-(rA) binding protein synthesis.

Alkaloids are known to have important role in medicinal chemistry due to their extensive biological activity. Especially noteworthy is the isoquinoline group of alkaloids that are widely distributed in several botanical families exhibiting myriad therapeutic applications.[8] Berberine chloride (Fig. 2), one of the most important member of the protoberberine group alkaloids, exhibits antisecretory, antiinflammatory, antibacterial, antimalarial as well as anticancer activities with significantly low cytotoxicity.[9–11] It has also been reported that berberine is useful in the treatment of dermal leishmaniasis, gastroenteritis in children, and cholera diseases.[12] The alkaloid induces apoptosis in HL-60 leukemia cells.[11] The molecular action of berberine underlying its biological properties was thought to be primarily DNA binding. It was established by us that berberine binds to DNA by partial intercalation exhibiting A–T base pair preference.[13–18] Nevertheless, a surprisingly stronger affinity to single stranded poly(rA) structure over B-DNA and t-RNA structures was also observed for berberine.[19] More recently discovered topoisomerase I and II poisoning activity and the A–T base specific DNA partial intercalation properties of the alkaloid are now thought to be responsible for its cytotoxic and pro-apoptotic action in the cell.[20] Both single and double stranded poly(rA) structures play important role in gene regulation in cells.[4–7] However their mode and mechanism of interaction with berberine is not yet clearly elucidated. The focus of our present study is to understand the complete molecular details of the binding process of berberine–poly(rA) complexation at the interaction site from both physicochemical and thermodynamic studies. Further, the interaction of berberine with double stranded poly(rA) structure is undertaken for comparative study.

2. Results

2.1. Spectral characteristics of poly(rA) structure

Figure 3 shows the absorption and circular dichroic (CD) spectra of poly(rA) at pH 7.1 and 4.5 for single stranded [hereafter ss poly(rA)] and double stranded [hereafter ds poly(rA)] structures. The absorption spectrum of ss poly(rA) at pH 7.1 has a maximum at 257 nm and on lowering the pH to ~4.5, this band maximum blue shifts to ~252 nm with a hypochromic effect. The absorption spectral characteristics of ss poly(rA) and ds poly(rA) are presented in Figure 3a and these are in conformity with our earlier observation.[19]

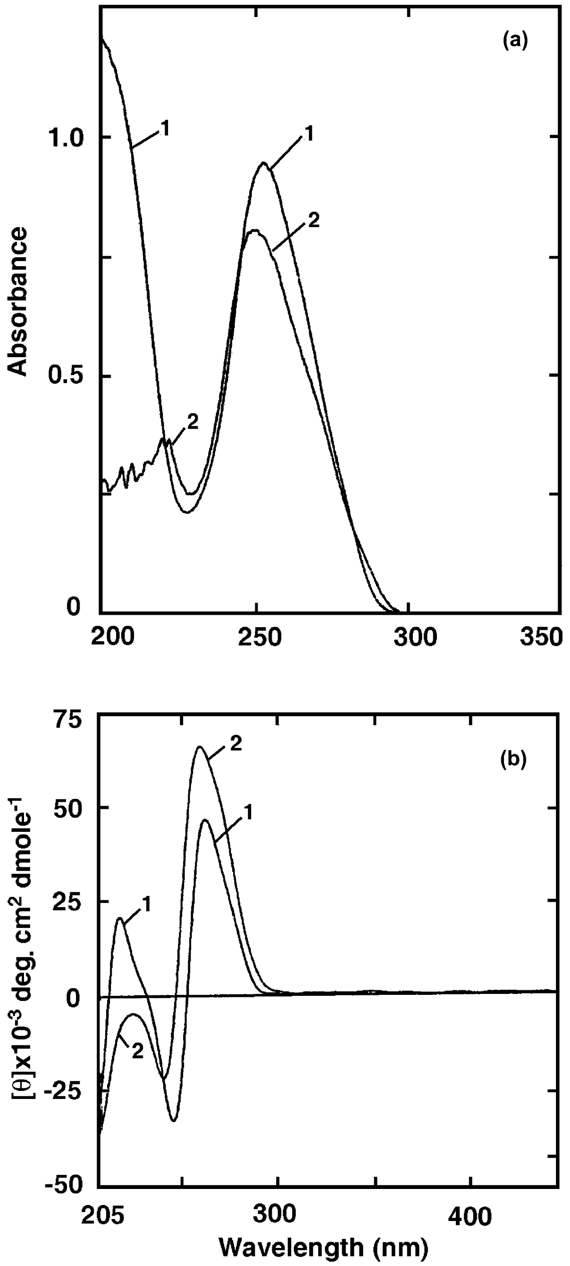

Figure 3. Absorption (a) and CD (b) spectra of (curve 1) ss poly(rA) (87.0 μM) and (curve 2) ds poly(rA) (85.0 μM) at 20 °C in buffer, pH 7.1 and 4.5, respectively.

The CD spectrum of the ss poly(rA) structure at pH 7.1 has a positive band around 265 nm and a strong negative band around 250 nm (Fig. 3b) followed by a positive band ~210 nm. When the pH is lowered to 4.5, the positive band enhances in ellipticity and blue shifts, while the negative band blue shifts and becomes weaker with a peak at around 242 nm. The positive band around 220 nm in the ss poly(rA) structure disappeared and a negative shoulder appeared around 230 nm (Fig. 3b). All these changes characterize the transition of poly(rA) from a single stranded helix to a double stranded helix.

2.2. Absorption spectral study

The effect of increasing concentration of ss poly(rA) on the absorption spectrum of berberine is presented in Figure 4a. The results show that bathochromic and hypochromic effects are observed on the absorption band of berberine until saturation was reached and these are characteristic features of aromatic chromophore (π–π*) interactions with the nucleic acid bases. The series of spectra exhibited three distinct isosbestic points at 445, 380, and 355 nm, respectively, indicating the equilibrium between bound and the free alkaloid molecules. The result of absorption titration was expressed in the form of Scatchard plot (inset, Fig. 4a), which indicated a non-linear non-cooperating binding process. Therefore the Scatchard plot was fitted to a theoretical curve drawn according to the excluded site model developed by McGhee and von Hippel[21] for a non-linear non-cooperative ligand binding. The best fit values of the binding parameters K' and n calculated from Eq. 1 are presented in Table 1. Figure 4b shows that the absorption spectrum of berberine does not alter significantly in presence of ds poly(rA) up to a *P*/*D* value of 10. This data clearly indicates that berberine does not bind to ds poly(rA).

2.3. Effect of ionic strength on berberine–ss poly(rA) complexation

The effect of ionic strength on the ss poly(rA)–berberine complexation was studied using absorbance spectroscopy at five different [Na$^+$] ion concentrations. It was observed that the extent of hypochromic and bathochromic effects in the absorption spectra decreased as the ionic strength increased (not shown). The binding parameters of the complexation obtained at the five [Na$^+$] ion concentrations are depicted in Table 2. In Figure 5 the variation of K' with [Na$^+$] ion concentration is presented in the log–log plot. It can be observed that the binding affinity varies linearly and falls by about 30 times on increasing the [Na$^+$] ion concentration from 4 to 50 mM, while the n value increase only 2 times under the identical condition.

2.4. Fluorescence spectral study

In aqueous buffer, berberine has an extremely weak fluorescence that gives an emission spectrum with a maximum around 545 nm when excited at 350 nm. The binding of ss poly(rA), however, remarkably enhanced the weak intrinsic fluorescence of berberine several fold. We observed a progressive enhancement of fluorescence intensity of berberine with increasing concentration of ss poly(rA). It can be seen from Figure 6a that at saturation, the steady state fluorescence of berberine was enhanced more than 90 times by ss poly(rA) indicating a remarkably strong association of the berberine chromophore with ss poly(rA) structure. The profound

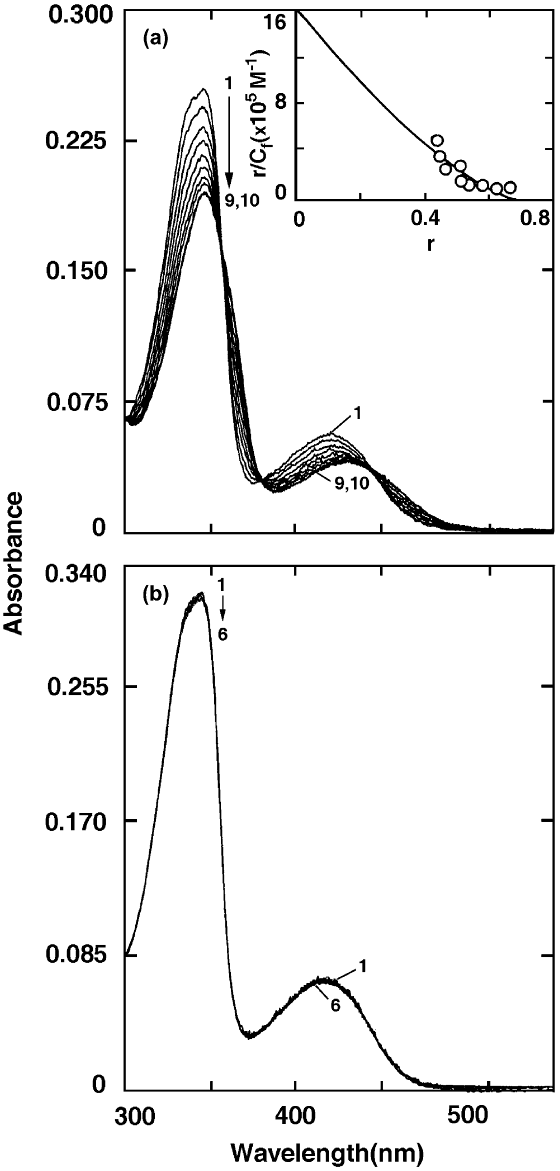

Figure 4. Representative absorption spectral changes of berberine in presence of (a) ss poly(rA) and (b) ds poly(rA). (a) Curves (1–10) denote absorption spectrum of (11.1 μM) berberine treated with 0, 3.34, 6.68, 10.02, 13.36, 16.70, 20.04, 23.38, 30.06, and 36.74 μM of ss poly(rA), respectively, in 4 mM BPES buffer pH 7.1 at 20 °C. Inset: representative Scatchard plot of binding of berberine to ss poly(rA). The solid line is the non-linear least square best fit of the experimental points to Eq. 1 with in the regions of the Scatchard plot ranging from 30% (lower limit) to 90% (upper limit). (b) Curves (1–6) denote absorption spectral changes of berberine (14.2 μM) treated with 0, 9.7, 29.1, 58.2, 87.3, and 145 μM of ds poly(rA) in 10 mM CP buffer pH 4.5 at 20 °C.

Table 1. Binding parameters for berberine–ss poly(rA) complexation in BPES buffer, 4 mM, pH 7.1 at 20 °C obtained from spectrophotometric study[a]

Binding parameter	Values
K' the intrinsic binding constant (M^{-1})	$1.90 \pm 0.02 \times 10^6$
n, the number of nucleotide occluded	1.50 ± 0.02

[a] Average of four determinations.

Table 2. Binding parameters for berberine–ss poly(rA) complexation obtained from spectrophotometric and spectrofluorimetric study in BPES buffer of various [Na^+] molarity at pH 7.1 at 20 °C[a]

[Na^+] molarity (mM)	Spectrophotometry		Spectrofluorimetry (quantum yield)[b]
	$K' \times 10^6$ (M^{-1})	n	
4	1.90 ± 0.02	1.50 ± 0.02	92.00
10	1.60 ± 0.03	1.75 ± 0.02	46.00
20	0.35 ± 0.01	2.25 ± 0.03	25.12
30	0.15 ± 0.01	2.35 ± 0.03	15.10
50	0.05 ± 0.01	2.50 ± 0.03	2.50

[a] Average of four determinations.

[b] Quantum yield value at saturation.

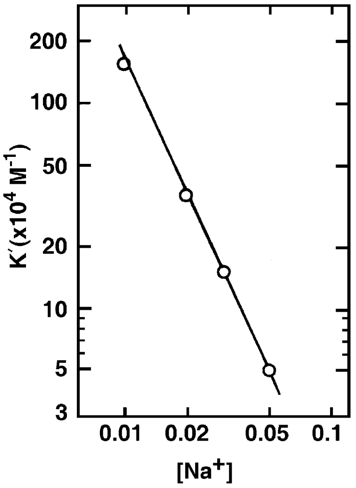

Figure 5. Log–log plot of intrinsic binding constant K' as a function of ionic strength [Na^+] for berberine binding to ss poly(rA) in BPES buffer pH 7.1 at 20 °C.

influence of salt on the association of berberine with ss poly(rA) was also observed in fluorescence studies and the results of such a study are depicted in Figure 6b–d. The enhancement of fluorescence intensity was significantly decreased with increasing salt molarity as evidenced from Figure 6. The quantitative data of fluorescence enhancement in terms of the quantum yield at different [Na^+] ion concentration is presented in Table 2.

A comparative fluorescence study (Fig. 7) on the interaction of berberine with ss poly(rU), ss poly(rC), B-form CT DNA, ds poly(rA), and ss poly(rA) shows that enhancement of berberine fluorescence intensity was very strong with ss poly(rA) and very weak with B-form CT DNA while practically no increase in its fluorescence intensity was observed in presence of ds poly(rA), ss poly(rU), or ss poly(rC). On comparison of the fluorescence data of berberine with ds poly(A)·poly(U) at a $P/D = 2.0$, at pH 7.1 and 4.5, it was revealed that the fluorescence intensity in these cases was lower than the values with ss poly(rA). This study clearly underscores the remarkably specific and higher affinity of binding of berberine to ss poly(rA) compared to other single stranded RNA, ds poly(rA), or B-form CT DNA and this is in confirmation with our earlier results.[19]

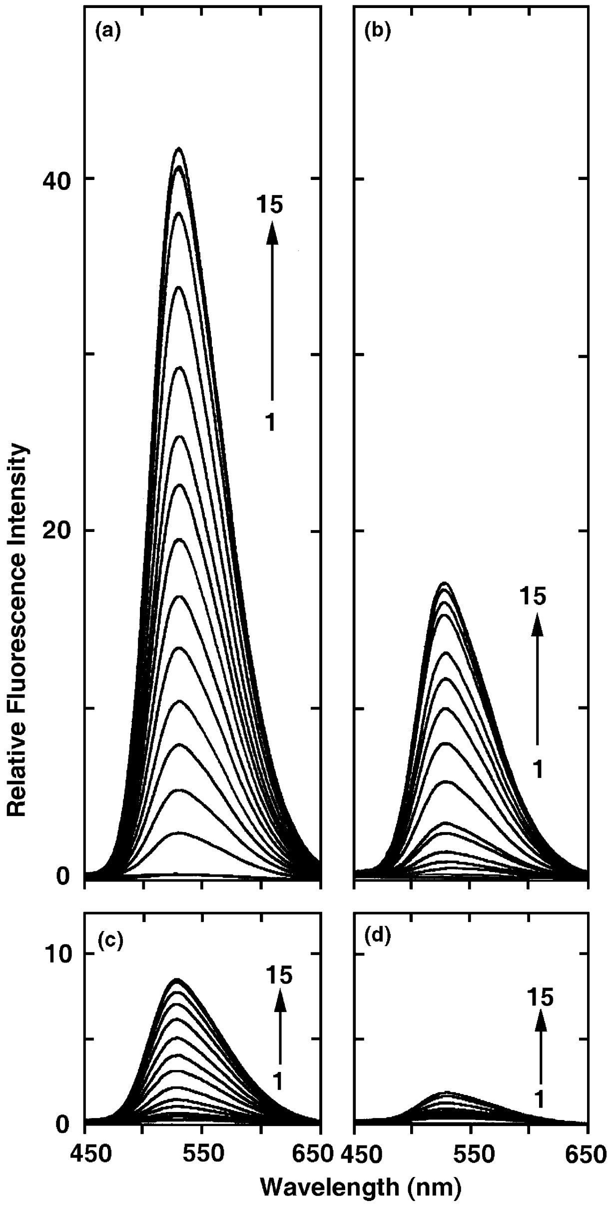

Figure 6. Representative steady state fluorescence emission spectrum of berberine (10.48 μM) treated with 0, 1.54, 3.01, 4.63, 6.18, 8.24, 10.30, 12.87, 15.45, 18.03, 21.63, 26.78, 33.47, 41.21, and 49.45 μM of ss poly(rA) in 4 mM (a), 10 mM (b), 20 mM (c), and 50 mM (d) of [Na^+] salt in BPES buffer of pH 7.1 at 20 °C.

2.5. Circular dichroic spectral study

Single stranded poly(rA) has characteristic CD spectrum with a sharp positive band at 265 nm and a negative band at 248 nm with a cross over around 255 nm (Fig. 8a). The intensity of the 265 nm band with an initial molar ellipticity of around 48,000 deg cm^2 $dmol^{-1}$ decreased when titrated with berberine and became around 10,000 deg cm^2 $dmol^{-1}$ at saturation. Correspondingly, the negative band also decreased, but the extent of change was smaller. Concomitant with the changes in the intrinsic CD in the UV region, there appeared a conservative extrinsic CD in the 300–400 nm region. The ellipticity of the extrinsic CD band increased as the bind-

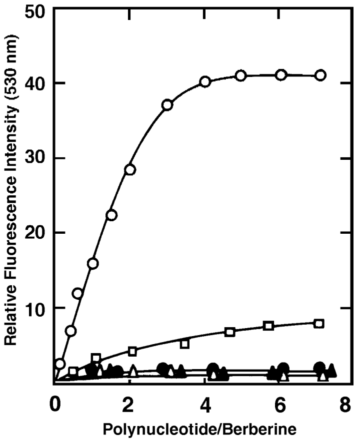

Figure 7. Increase of steady state fluorescence intensity of berberine (10.5 μM) at 530 nm in presence of increasing concentration of (○—○) ss poly(rA); (□–□) CT DNA; (△–△) ss poly(rU); (▲–▲) ss poly(rC) in buffer pH 7.1 and (●—●) ds poly(rA) in buffer pH 4.5 at 20 °C, excited at 350 nm. Each point was an average of four sets of experiments.

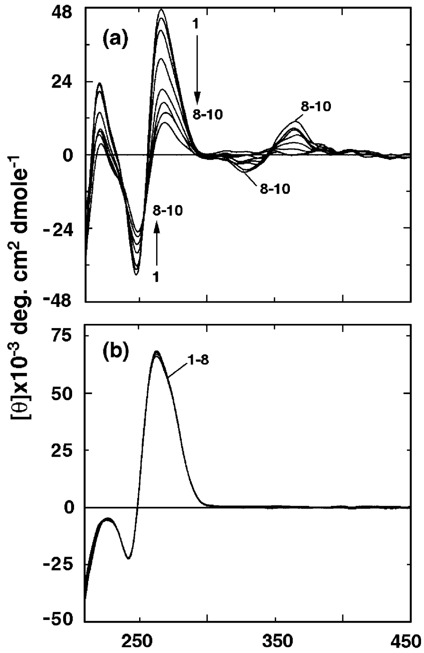

Figure 8. Representative CD spectra resulting from interaction of berberine with (a) ss poly(rA) and (b) ds poly(rA) structures. (a) Curves (1–10) denote 61.51 μM ss poly(rA) treated with 0, 3.06, 6.15, 12.30, 18.45, 24.60, 30.75, 36.90, 43.05, and 48.25 μM of berberine, respectively, in 10 mM BPES buffer of pH 7.1 at 20 °C. (b) Curves (1–8) denote 61.51 μM ds poly(rA) treated with 0, 3.07, 6.15, 12.30, 18.45, 24.60, 30.75, and 36.90 μM of berberine, respectively, in 10 mM CP buffer of pH 4.5 at 20 °C.

ing progressed. Figure 8b shows that the CD of ds poly(rA) is not affected with increasing concentration of berberine and also generation of extrinsic CD was not observed.

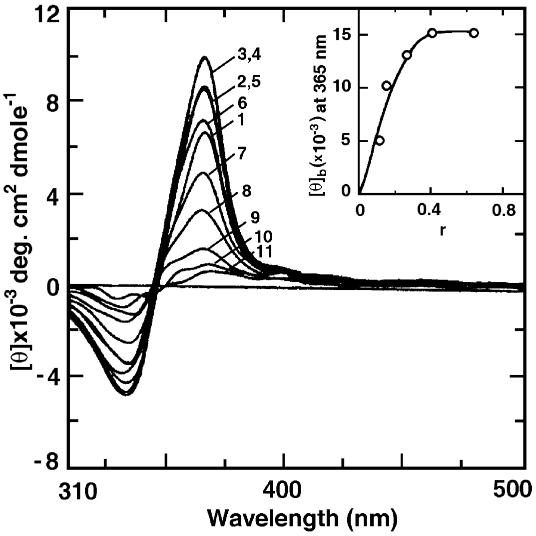

Figure 9. Extrinsic CD spectrum of berberine (50 μM) treated with 1000, 850, 723, 614, 522, 443, 355, 266, 200, 140, and 98 μM of ss poly(rA), respectively, as indicated by curves (1–11) in BPES buffer of pH 7.1 at 20 °C. Inset: variation of molar ellipticity $[\theta]_b$ against alkaloid bound (*r*). $[\theta]_b$ is based upon the bound berberine concentration at 365 nm and was calculated according to $[\theta]_b = 100{\cdot}\theta/C_b{\cdot}l$ where θ is the observed ellipticity in degrees, C_b is the molar concentration of bound berberine and l is the path length in cm. $[\theta]_b$ is expressed in terms of $\deg \mathrm{cm}^2 \mathrm{dmol}^{-1}$. The values of *r* for each berberine–ss poly(rA) complex was obtained directly from concurrently performed spectrophotometer titration. Each value of $[\theta]_b$ was the average of three sets of experiments.

The extrinsic CD changes on the interaction of berberine with ss poly(rA) was studied keeping a fixed concentration of berberine and varying the concentration of poly(rA). The result of such study is presented in Figure 9. The extrinsic CD spectrum at a *P*/*D* 20 denoted by spectrum 1 is characterized by a conservative pair of CD bands with maxima centered at 370 and 335 nm, respectively, and with a cross over around 348 nm. The molar ellipticity of the positive extrinsic CD bands increased on decreasing the *P*/*D*. A maximum in the effect was achieved at a *P*/*D* of 13, thereafter which the ellipticity decreased. The series of spectra exhibited an isoelliptic point at 348 nm, the cross over point. A plot of molar ellipticity values at 365 nm in terms of bound alkaloid ($[\theta]_b$) against *r* is presented in the inset of Figure 9.

2.6. Denaturation and renaturation study

The effect of temperature on ss poly(rA) structure in absence and presence of berberine obtained from spectrophotometric and circular dichroic studies is shown in Figure 10. It can be seen that ss poly(rA) structure shows the process of denaturation (i.e., structural transition from helix to random coil) with increasing temperature from 20 to 60 °C and a complete renaturation with decrease in temperature to 20 °C. This clearly showed a complete transition of the helical structure of ss poly(rA) to random structure and back indicating the native integrity of poly(rA) molecules used in this study. But in presence of berberine, both the denaturation and renaturation process of ss poly(rA) structure were significantly affected as evidenced from the results depicted in Figure 10b. The effect of increase of temperature on ss

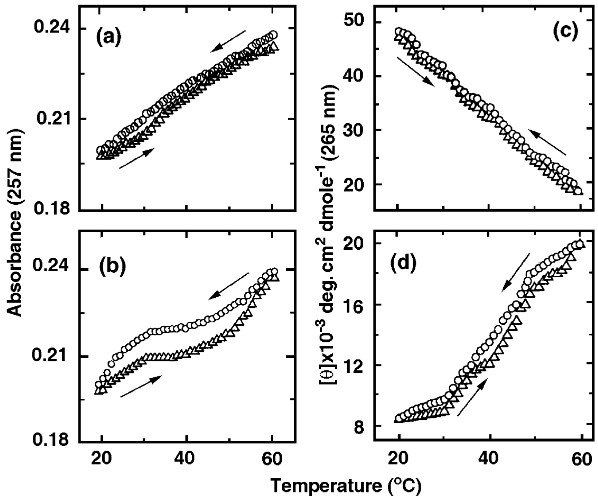

Figure 10. Spectrophotometric measurements on heating (△–△) and cooling (○—○) of 20.1 μM ss poly(rA) in absence (a) and in presence (b) of berberine ($D/P = 0.5$) in BPES buffer of pH 7.1. The same concentration of berberine (12.06 μM) was kept in control and sample cuvette to eliminate the contribution of absorption of berberine at different temperature. Spectropolarimetric measurements on heating (△–△) and cooling (○—○) of 61 μM of ss poly(rA) in presence (c) and in absence (d) of berberine ($D/P = 0.8$) in BPES buffer, pH 7.1. The arrows indicate the direction of heating and cooling process.

poly(rA) shows significant decrease of ellipticity of CD band at 265 nm up to 60 °C and a complete reverse back to the native structure on cooling as shown in Figure 10c. In presence of berberine also the increase of CD changes at 265 nm on heating was completely reversible, unlike that observed in UV melting study on cooling (Fig. 10d). This is an unusual observation. If the denaturation of poly(rA) occurs in presence of berberine, the results should have been a quenching of intensity of CD band at 265 nm with increasing temperature. It seems that berberine binding is more favored at higher temperatures up to 60 °C and it stabilizes rather than its transition from helical to coil structure indicating strong binding of berberine to single stranded poly(rA) structure. Further studies on the effect of temperature on berberine–ss poly(rA) complex was performed by monitoring changes in the extrinsic CD pattern as well as the ellipticity change at 365 nm at different temperature. The results are presented in Figure 11. The positive ellipticity of the complex at 365 nm decreased slowly up to 30 °C, while thereafter the change was rapid.

2.7. Viscometric study

The mode of binding of berberine to ss poly(rA) helix was investigated from viscosity studies (Fig. 12). The relative specific viscosity of the poly(rA)–berberine complex increased as the *D*/*P* increased suggesting the intercalation of the alkaloid into the helical organization of the ss poly(rA) structures. No viscosity enhancement was, however, observed in ds poly(rA) in presence of increasing concentration of berberine (Fig. 12). This data clearly established that berberine does not form a complex with ds poly(rA).

2.8. Thermodynamics of the interaction

To evaluate the thermodynamics of berberine–ss poly(rA) complexation, spectroscopic binding studies were performed at four different temperatures namely 15, 20, 25, and 30 °C at a constant [Na$^+$] molarity. The binding parameters evaluated at these temperatures in turn enabled the determination of the thermodynamic parameters of the binding. Table 3 depicts the binding data obtained from spectrophotometric titration at the four different temperatures. It can be seen that the bind-

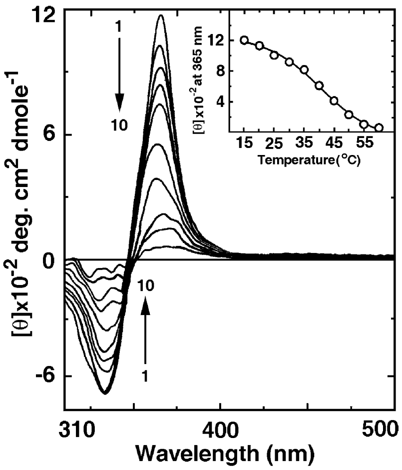

Figure 11. Temperature dependence of the extrinsic CD of berberine (50 μM) treated with a ss poly(rA) (100 μM) in BPES buffer of pH 7.1. Curves 1–10 denote spectra at every 5 °C interval between 15 and 60 °C, respectively. The expressed molar ellipticity is based on the input berberine concentration. Inset: a plot showing the variation of molar ellipticity [θ] at 365 nm as a function of temperature. Each value of [θ] was the average of three set of experiments.

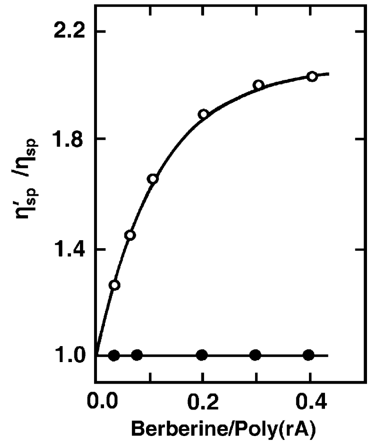

Figure 12. A plot of change of relative specific viscosity of (○—○) ss poly(rA) and (●–●) ds poly(rA) with increasing concentration of berberine in 10 mM buffer pH 7.1 or 4.5 at 20 °C. The relative specific viscosity was calculated from the equation $\eta'_{sp}/\eta_{sp} = [t_2\ \text{(complex)} - t_1]/[t_2\ \text{(control)} - t_1]$, where η_{sp} and η'_{sp} are the specific viscosity of native ss poly(rA) or ds poly(rA) in absence and in presence of berberine; t_2 (complex) and t_2 (control) are the efflux times of complex and control solution and t_1 is the same for buffer solution. The concentrations of ss poly(rA) and ds poly(rA) were 433 and 340 μM, respectively.

Table 3. Binding parameters of berberine–ss poly(rA) complexation obtained from spectrophotometric experiments at various temperatures in BPES buffer, 10 mM [Na$^+$], pH 7.1 at 20 °C[a]

Temperature, °C	Spectrophotometry	
	$K' \times 10^6$	n
15	1.20 ± 0.02	1.60 ± 0.02
20	1.60 ± 0.02	1.75 ± 0.02
25	2.00 ± 0.03	1.39 ± 0.01
30	2.80 ± 0.03	1.10 ± 0.01

[a] Average of three determinations.

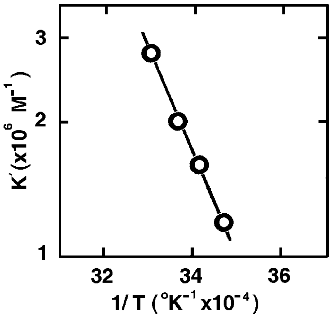

Figure 13. van't Hoff plot for the complexation of berberine with ss poly(rA). The data were fitted to straight line with correlation coefficient of 0.981.

Table 4. Thermodynamic parameters of berberine–ss poly(rA) complexation obtained from spectrophotometric experiments[a]

Parameter (20 °C)	Value
ΔG^0	$-8.28\,\mathrm{kcal\,mol^{-1}}$
ΔH^0	$+8.573\,\mathrm{kcal\,mol^{-1}}$
ΔS^0	$+57.5\,\mathrm{cal\,deg^{-1}\,mol^{-1}}$

[a] Average of three determinations.

ing constants (K') show unusual temperature dependence. The binding constant increased with increasing temperature, while the number of nucleotide occluded sites (n) decreased. This study further indicated that within the temperature range studied the complexation is more favored at 30 °C compared to that at 15 °C. These results are again in conformity with the CD results (Fig. 10d) of berberine–ss poly(rA) complex where berberine bound more ss poly(rA) with increasing temperature. The van't Hoff plot for the binding of berberine to ss poly(rA) is depicted in Figure 13. The data fits to a straight line indicating a small value of heat capacity change. The values of the thermodynamic parameters are depicted in Table 4. It can be seen that the binding of berberine to ss poly(rA) is characterized by positive enthalpy and positive entropy changes.

3. Discussion

Results obtained previously from extensive spectroscopic and viscometric studies have revealed that berberine forms a molecular complex with B-form DNA by mechanism of partial intercalation exhibiting specificity toward AT base pair sequences.[13–18] This was further supported by biochemical and molecular modeling studies by other workers.[22] We had also reported for the first time that berberine binds to ss poly(rA) structure more strongly than B-DNA and its affinity in terms of binding constant was about 12 times higher than B-DNA.[19] It has been reported that berberine inhibits protein synthesis much more than DNA synthesis in S180 tumor cells.[23] The greater effective binding capacity of berberine toward ss poly(rA) structure has thus been correlated with greater inhibition of protein synthesis in eukaryotic cells.[19]

The strong interaction of berberine with ss poly(rA) structure was evident from the observation of typical bathochromic and hypochromic effects in the absorption spectra, remarkable enhancement of fluorescence intensity, increase in fluorescence quantum yield, significant perturbation of CD spectra, generation of extrinsic CD bands, increase in viscosity and sign and magnitude of the thermodynamic parameters. Berberine shows highest affinity for ss poly(rA) and does not associate with ds poly(rA), ss poly(rU), or ss poly(rC) structures. The bathochromic and hypochromic effects of berberine–ss poly(rA) complex are similar to that observed for B-DNA–berberine complexation[15,17] and are also in agreement with its complexation with other ligands.[24–26] Again, the order and magnitude of intrinsic binding constant obtained from spectrophotometric study are similar to that was observed in an earlier study.[19] The results obtained from denaturation and renaturation study in UV melting also support that berberine forms a strong complex with ss poly(rA) structure and inhibits significantly its normal renaturation process. Temperature effects on extrinsic CD band maximum [θ_{365} nm] upon binding of berberine to ss poly(rA) reflected that the asymmetric arrangement of berberine decreased slowly up to a temperature of 30 °C while thereafter the asymmetry decreased rapidly due to the release of berberine molecules from the poly(rA) helix.

The binding of berberine was greatly influenced by the ionic strength of the medium. The value of the binding constant (K') with ss poly(rA) decreased almost 30 times when a 5-fold increment in the [Na$^+$] ion concentration was effected, while variation of n values with increasing salt molarity was marginal. These results clearly emphasized the substantial role of the electrostatic component in the binding process. Similar results were also reported in case of berberine and several other cationic ligands interacting with B-DNA.[14,27–31]

The extrinsic CD of berberine bound to ss poly(rA) suggested that the alkaloid molecule was inserted between neighboring adenine bases through stacking of berberine with bases on the same chain and are in conformity to our results as proposed earlier.[19] Since the optical activity is induced on berberine bound by ss poly(rA), it is perceived that the bound berberine forms a right handed helical conformation with the nearby bound berberine molecules on the right handed ss poly(rA) helix due to exciton coupling of berberine absorption in this region. This asymmetry leads to a conservative extrinsic CD spectrum. It can also be seen that the magnitude of

$[\theta]_b$ increases as a function of r and attains a maximum where the bound berberine is maximum.

The most important evidence for the intercalative mode of binding was obtained from the viscosity measurements with ss poly(rA) structure. The present findings on the increase in specific viscosity of the helical ss poly(rA) structure resembles to those of its complexation with B-DNA structure[13] indicating that the strong binding of berberine is responsible for helical stability and an elongation of ss poly(rA) structure on complexation with berberine. The binding mode is proposed to be in favor of partial intercalation and may be significantly different from the original intercalation model proposed by Lerman,[32] which assumes that planner ligand molecule is sandwiched between hydrogen-bonded base pairs of double stranded DNA.

The temperature dependence of the binding of berberine to ss poly(rA) structure was used to derive the thermodynamic parameters. Conceptually, the thermodynamic parameters describing the binding reactions may be divided into three contributions. First is the contribution from the molecular interactions between the bound ligand and nucleic acid binding site as a result of hydrogen bonding and hydrophobic interactions. Next are the contributions arising from the conformational changes in either the nucleic acid or the drug upon binding. Finally, there are contributions, which may be coupled processes like ion release, proton transfer, or changes in the water of hydration. Present data (Table 4) revealed that the interaction of berberine with ss poly(rA) structure is endothermic and the binding process is entropy driven. The intermolecular interaction is characterized by a positive enthalpy change and a positive entropy change. All of these strongly suggest binding events, where water molecules play an important role. In drug–nucleic acid complex water molecules either add stability by participating in the intermolecular interactions or can change the state of hydration of nucleic acid structure on binding. In our study the thermodynamic characteristics of berberine–ss poly(rA) complex show large positive entropy change reflecting release of water molecules more compared to the binding of berberine to double stranded B-form DNA.[27] However, conformational change of ss poly(rA) structure on binding to berberine cannot be ruled out. The positive enthalpy and the positive entropy values characterize gross changes in the overall binding process and both favors the tight binding of berberine at the interaction site. Similar characteristics were also observed in many B-DNA–ligand complexation.[33–35]

The study on the interaction of berberine with ds poly(rA) revealed that it does not alter (i) the absorption and fluorescence spectrum of berberine up to a P/D value of 10 and (ii) A-form CD spectrum of ds poly(rA), and also (iii) the viscosity of ds poly(rA). The study has, thus, clearly demonstrated that berberine does not bind to ds poly(rA) structure. It is known that ds poly(rA) plays significant role in intracellular process of eukaryotic cells as termination of mRNA + poly(rA) synthesis.[7] But the present observation shows that berberine may not interfere or inhibit this process in eukaryotic cells.

The transcription process in eukaryotic cells as depicted in Figure 1 showed the role of ss poly(rA) in the post transcriptional modification for protein synthesis. Thus, if any compound binds strongly to poly(rA) tail in the transcriptional process, it should inhibit protein biosynthesis in cells. In this context it is interesting to note that Creasey[23] has reported inhibition of protein synthesis (96.8%), RNA synthesis (86.7%), and DNA synthesis (51.6%) in S180 tumor cells in presence of 10.5 μM berberine. Present results clearly show that berberine molecules assume a helical arrangement after complexation with ss poly(rA) as evidenced from both intrinsic and extrinsic CD studies (Figs. 8 and 9) and the binding process is entropy driven, which demonstrate that berberine very strongly associates to ss poly(rA) than B-form DNA. This strong binding of berberine to ss poly(rA) structure clearly support the greater inhibition of protein synthesis in S180 tumor cells.[23] As ss poly(rA) plays significant role in control of eukaryotic cell processes, our results suggest a potential mechanism by which the alkaloid might inhibit the process of gene expression and gene transcription probably leading to its usefulness as an antitumor alkaloid.

4. Materials and methods

Berberine chloride (Lot no. 119H0687) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Its purity was verified by thin layer chromatography and melting point determination. Since no impurities were seen, no further purification was done. Poly(rA) (Lot no. 32H4016) calf thymus (CT) DNA, (Lot no. 73H9563), poly(rU) (Lot no. 119H4053), poly(rC) (Lot no. 128F4015), and poly(A)·poly(U) (Lot no. 109H4053) were products of Sigma Chemical Company and were used as such. Stock solutions of all polynucleotides were made in 10 mM BPES buffer (1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, and 6 mM NaCl) pH 7.1. Concentration of each polymer was determined spectrophotometrically using the molar extinction coefficients, $\varepsilon_{257} = 1.0 \times 10^4\,M^{-1}cm^{-1}$ for poly(rA), $\varepsilon_{260} = 6.6 \times 10^3\,M^{-1}cm^{-1}$ for CT DNA, $\varepsilon_{261} = 9.5 \times 10^4\,M^{-1}cm^{-1}$ for poly(rU), $\varepsilon_{269} = 6.2 \times 10^4\,M^{-1}cm^{-1}$ for poly(rC) and $\varepsilon_{261} = 7.14 \times 10^3\,M^{-1}cm^{-1}$ poly(A)·poly(U), respectively. The alkaloid solution was prepared afresh each day and the concentration was determined using a molar extinction coefficient (ε) of 22,500 $M^{-1}cm^{-1}$ at 344 nm. The alkaloid obeyed Beer's law in the concentration range employed in our study. The double stranded conformation of poly(rA) was prepared by adding poly(rA) solution slowly into buffer of pH 4.5 and waiting for at least 2 h for the transition to be complete.

Binding experiments with ss poly(rA) and other polymers at neutral pH were performed in BPES buffer, pH 7.1 of different $[Na^+]$ ion concentrations while for ds poly(rA), citrate–phosphate (CP) buffer containing 5 mM Na_2HPO_4 adjusted to pH 4.5 with citric acid was employed. pH measurements were made on an EC digital pH meter (Electronics Corporation, India) with an accuracy of ±0.02 units. Deionized and triple distilled water and analytical grade reagents were used. All the buffer solutions were passed through Millipore filters of 0.45 μM.

4.1. Absorption spectral study

Absorption spectral measurements were performed on a Shimadzu UV 260 spectrophotometer (Shimadzu Corporation, Japan) in matched quartz cuvettes of 1 cm path length as described previously.[36,37] Spectrophotometric titrations were performed by keeping a constant concentration of berberine while varying the poly(rA) concentration as described earlier.[19]

4.2. Evaluation of binding parameters

The results of absorption titration measurement were expressed in the form of Scatchard plots as r/C_f versus r where r is the number of molecules of alkaloid bound per mole of nucleotide and C_f is the molar concentration of the free alkaloid. The data were further analyzed by the excluded site model[21] for non-linear non-cooperative ligand binding phenomenon using the following equation:

$$r/C_{\mathrm{f}} = K'(1-nr)[(1-nr)/1-(n-1)r]^{n-1}, \qquad (1)$$

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[38] version 1.2 that works on an algorithm that determines the best fit parameters to Eq. 1 as described earlier.[36,37]

4.3. Thermal melting study

Temperature dependent absorption spectral profiles of ss poly(rA) in absence and presence of berberine were recorded on the Shimadzu UV 260 unit equipped with a temperature programmer (KPC-5) and controller (SPR-5) in stoppered quartz cuvettes of 1 cm path length monitoring the absorbance change at 257 nm on increasing (denaturation) and lowering (renaturation) the temperature. The temperature was scanned using a heating rate of either 0.5 or 1 °C/min. In temperature dependent experiments, the same amount of berberine was present in control as well as the sample cuvette that eliminated the absorbance of berberine at 257 nm due to temperature effect.

4.4. Fluorescence study

The fluorescence measurements were recorded on a Hitachi model F4010 spectrofluorimeter (Hitachi Ltd, Tokyo, Japan) as described earlier,[39] where a fixed concentration of the alkaloid was titrated with increasing concentrations of poly(rA) in fluorescence free quartz cuvettes of 1 cm path length. Steady state fluorescence quantum yield was calculated using the following equation as described earlier:[37,39]

$$\phi_{\mathrm{s}} = (F_{\mathrm{s}}\varepsilon_{\mathrm{q}}C_{\mathrm{q}}/F_{\mathrm{q}}\varepsilon_{\mathrm{s}}C_{\mathrm{s}})0.55, \qquad (2)$$

where, F denotes the integral area of the fluorescence in arbitrary units, s and q denote sample and quinine sulfate in 0.1 N H_2SO_4, respectively. ε is at the molar extinction coefficient and C represents the corresponding concentration.

4.5. Spectropolarimetric study

Circular dichroism (CD) spectra were recorded on a Jasco J 720 spectropolarimeter as described earlier.[39,40] The denaturation and renaturation spectra of ss poly(rA) in absence and in presence of berberine were taken at 2° intervals. In practice samples were equilibrated for at least 5 min at each temperature before the readings were taken. A thermocouple probe inserted into the sample showed that these hold times were sufficient for thermal equilibration. The reported spectra are averages of four successive scans and are base line corrected and smoothed. The ellipticity values are expressed in terms of either per nucleotide phosphate or per bound alkaloid and the molar ellipticity (θ) was expressed in deg cm^2/dmol.

4.6. Viscometric study

Viscosity measurements were performed using a Cannon-Manning Type 75 semimicro viscometer mounted vertically in a constant temperature bath (Cannon Instruments Co., State College, PA, USA) maintained at 20 ± 0.05 °C as described previously.[41] Flow times of sample alone and sample mixed with different ratios of alkaloid were measured by an electronic stopwatch model HS-30W (Casio Computer Co. Ltd, Japan) with an accuracy of 0.01 s. Viscosity experiments were carried out with 433 and 340 μM of ss and ds poly(rA) sample, respectively.

4.7. Thermodynamic study

To evaluate the thermodynamic parameters, the binding constants were determined at 15, 20, 25, and 30 °C from absorption measurements. The binding constants were determined either from a complete titration at a particular temperature or by increasing the temperature of a sample containing a fixed ratio of the poly(rA)/berberine (P/D) and the thermodynamic parameters (ΔG^0, ΔH^0, and ΔS^0) were estimated as described earlier.[27,31]

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