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Thionine Interaction to DNA: Comparative Spectroscopic Studies on Double Stranded Versus Single Stranded DNA

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Abstract Interaction of thionine with double stranded and single stranded calf thymus DNA has been studied by absorbance, fluorescence, competition dialysis, circular dichroism and isothermal titration calorimetry. Binding to the native double stranded DNA conformation induced strong quenching in fluorescence spectrum of thionine. Linear Scatchard plots indicated the binding to be of one type and the affinity values evaluated to be of the order of 10^5 M^{-1} with double stranded DNA. Fluorescence quenching was much weaker with single stranded DNA and the binding affinity was about one order lower. Ferrocyanide quenching studies revealed that the fluorescence emission of dye molecules bound to the double stranded DNA was quenched much less compared to those bound to the single stranded DNA. Furthermore, there was significant emission polarization for the bound dye molecules and strong energy transfer from the DNA base pairs to the dye molecules indicating intercalative binding to ds DNA. Salt dependence of the binding phenomenon revealed that electrostatic forces played a significant role in the binding process. The intercalation of the dye molecules to double stranded DNA and simple stacking to single strands was proved from these fluorescence techniques. Support to the fluorescence results have been derived from absorption, circular dichroic and dialysis results. Calorimetric studies suggested that the binding to ds DNA conformation was both enthalpy and entropy favoured while that to ss DNA was predominantly entropy favoured.

Keywords Thionine · DNA binding · Spectroscopy · Competition dialysis · Calorimetry

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Introduction

Studies on the binding aspects of small molecules with DNA have been an interesting topic for several researchers across many laboratories in the world for a long time [1–6]. DNA binding studies have remarkable relevance and implications in drug design aspects and cancer chemotherapy. A strong and highly sequence/structural specific binding with the genomic DNA and consequent structural damage may be effective in preventing the arrest of cell division. Planar small molecules bind DNA by intercalative interaction and this has been the subject matter of a number of investigations in the past [1, 6, 7]. Although a large volume of data is available, a clear cut understanding on the nature, specificity and energetics of the interaction and the use of such data for the development of effective DNA targeted chemotherapeutic agents remained an elusive goal. Consequently, the molecular aspects of small molecule-DNA interaction continues to be an interesting and highly challenging field of research activity.

Thionine (Fig. 1), a tricyclic heteroaromatic molecule belongs to the phenothiazine group of dyes. It has attracted recent attention for use in semiconductors, as energy sensitizer, as a probe for investigating various microenvironments including micells and polymeric matrices in the preparation of functionalized nanocomposite materials and in preparing high quantum efficiency photoelectrochemical cells [8–13]. The DNA binding aspects of thionine has been

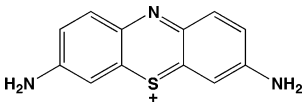

Fig. 1 Chemical structure of thionine

known in terms of intercalative binding [7, 14]. Thionine exhibits guanine-cytosine specifictiy in its DNA binding [15–17] More recently the use of thionine as charge neutralizer for impedance based DNA biosenser employing its binding to single and double stranded DNA has been reported [18]. Although binding to duplex DNA is known, the binding aspects of thionine to single stranded DNAs are not known. Herein, we use extensive spectroscopic techniques for the characterization of the binding reaction between thionine to double stranded and single stranded calf thymus DNA. Here we have exploited the strength of various fluorimetric tools like steady state, Stern–Volmer quenching, polarization anisotropy, quantum yield, energy transfer from base pairs to dye etc to characterize and analyze the interaction of thionine to calf thymus DNA in the double stranded and single stranded helical conformations. The fluorimetric data has also been supplemented by absorbance, circular dichroism and calorimetric data.

Experimental

Apparatus

Absorbance measurements were done on a Jasco V 660 spectrophotometer (Jasco International Co. Ltd, Tokyo, Japan) and melting profiles were obtained on a Shimadzu Pharnaspec 1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) as described in details elsewhere [16, 19].

Spectrofluorimetric measurements were performed either on a Hitachi F4010 unit (Hitachi Ltd. Tokyo, Japan) or Shimadzu RF-5301PC unit using 1 cm path length quartz cuvettes. An excitation and emission band pass of 5 nm were used. Polarization measurements were performed on the Hitachi unit provided with the excitation and emission polarizers as reported earlier [20, 21].

Circular dichroic (CD) spectra were acquired on a PC controlled spectropolarimeter Jasco J815 model equipped with a Jasco temperature programmer (model PFD 425L/ 15) at 20±0.5 °C as described in details elsewhere [16, 22].

All isothermal titration calorimetry experiments were performed using a MicroCal VP-ITC unit (MicroCal, Inc., Northampton, MA, USA) using protocols developed in our laboratory [23, 24].

Reagents

Double stranded (ds) calf thymus (CT) DNA was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). The absorbance ratio of the sample A_{260}/A_{280} was in the range 1.80–1.90 and indicated the protein free nature of the sample. A molar extinction coefficient (ε) value of 6,600 M^{-1} cm^{-1} was used for estimating the concentration by absorbance measurements at 260 nm. Single stranded (ss) CT DNA was prepared by heating the ds sample of CT DNA in presence of formaldehyde for 10 min. and plunging in ice bath as reported in the literature [25]. All stock solutions were stored in a refrigerator at 4 °C. Thionine acetate (hereafter described as dye or ligand) obtained from Sigma-Aldrich were used without further purification. Solutions were freshly prepared each day in the buffer and kept protected in the dark till use. A molar extinction coefficient value of 54,200 M^{-1} cm^{-1} at 598 nm was used for estimation of the concentration of thionine solutions [16]. Beer's law was found to be obeyed in the concentration range used in this study. All experiments were conducted in 50 mM cacodylate buffer, pH 7.2, prepared in deionised and triple distilled water. Buffer solutions were filtered through Millipore filters of 0.22 μm. All other chemicals and reagents used were of analytical grade or better.

Methods

Absorption Spectra

All absorbance spectra were measured on a Jasco V 660 double beam, double monochromator spectrophotometer (Jasco International Co, Tokyo, Japan) at 20± 0.5 °C in 1 cm path length quartz cuvettes using the methodologies described in details earlier [16, 19]. Melting profiles of the DNA samples were performed in a Shimadzu Pharmaspec 1700 unit (Shimadzu Corporation, Kyoto, Japan) equipped with a TMSPC melting accessory in eight chambered quartz cuvettes as described earlier [16, 19]. The melting temperature (T_m) of the double stranded CT DNA under the present experimental conditions was 79±1 °C.

Steady State Fluorescence Spectra

Steady state fluorescence measurements were performed on a Shimadzu RF5301PC spectrofluorimeter in fluorescence free quartz cuvettes of 1 cm path length [20].

Fluorescence Quenching Measurements

Fluorescence quenching studies were carried out with the anionic quencher $K_4[Fe(CN)_6]$. The quenching experiments were performed by mixing, in different ratios, two solutions, one containing KCl, the other containing $K_4[Fe(CN)_6]$, in addition to the normal buffer components, at a

fixed total ionic strength. Fluorescence quenching experiments were performed at a constant P/D (DNA nucleotide phosphate / dye molar ratio) monitoring fluorescence intensity as a function of changing concentration of the ferrocyanide as described previously [22]. At least four measurements were taken for each set and averaged out. The data were plotted as Stern–Volmer plots of relative fluorescence intensity (F_o/F) versus $[Fe(CN)_6]^{4-}$ concentration according to the Stern–Volmer equation

$$F_o/F = 1 + K_{SV}[Q] \quad (1)$$

where F_o and F denote the fluorescence emission intensities in the absence and presence of the quencher and [Q] is the quencher concentration. K_{SV} is the Stern–Volmer quenching constant, which is a measure of the efficiency of fluorescence quenching by the quencher.

Measurement of Fluorescence Energy Transfer

Energy transfer from the DNA base pairs to the bound dye molecules was measured from the excitation spectra of the DNA-dye complexes in the wavelength range 220–310 nm [26, 27]. Excitation spectra were recorded keeping the emission wavelength at 615 nm for the dye. The ratio $Q = q_b/q_f$, where q_b and q_f are the quantum efficiencies of bound and free dye, respectively, was calculated for each wavelength using the equation $Q = q_b/q_f = I_b\varepsilon_f/I_f\varepsilon_b$, where I_b and I_f are the fluorescence intensities in the presence and absence of the DNA, respectively, and ε_b and ε_f are the corresponding molar extinction coefficients of the dye. A plot of the ratio of Q_λ/Q_{310} against wavelength was made. Since DNA has very little absorbance at 310 nm this was chosen as the normalization wavelength.

Fluorescence Polarization

Fluorescence polarization measurements of thionine and its complexes with the DNAs were carried out as per Larsson and colleagues [28] on the Hitachi F4010 spectrofluorimeter as described previously [21]. Steady state polarization anisotropy 'A' is defined as

$$A = (I_{vv} - I_{vh}G)/(I_{vv} + 2\,I_{vh}G) \quad (2)$$

where G is the ratio I_{hv}/I_{hh} used for instrumental correction. I_{vv}, I_{vh}, I_{hv} and I_{hh} represent the fluorescence signal for excitation and emission with the polarizer positions set at (0°, 0°), (0°, 90°), (90°, 0°) and (90°, 90°), respectively.

Competition Dialysis Assay

Competition dialysis assay was carried out in general following the procedures of Chaires and colleagues [29, 30]. A 0.5-mL solution of each of the DNA solution (at identical concentration of 150 μM in base nucleotide units) was dialyzed in separate 0.5 mL slide-a-lyzer mini dialysis unit (Pierce Chemical Co, IL, USA) against a 1-μM dialysate solution of the dye solution under stirring in a 200-mL glass beaker at room temperature (20±1 °C). At the end of the equilibration period (24 h), the total concentration of thionine (C_t) that enters the membrane into the dialysis unit was determined spectrophotometrically after dissociating the complex by adding appropriate volume of a 10% (*w*/*v*) of sodium dodecyl sulphate (SDS) to make a final concentration of 1% (*w*/*v*) allowing to equilibrate for 2 h. The concentration of the free thionine (C_f) was determined spectrophotometrically using an aliquot of the dialysate solution although its concentration did not vary from the initial 1-μM concentration. The amount of thionine bound to the nucleic acids (C_b) was determined by difference, ($C_b = C_t - C_f$). This data were then used to calculate the apparent binding constant (K_{app}) using the relationship

$$K_{app} = C_b/(C_f)(S_{total} - C_b) \quad (3)$$

where S_{total} = 150 μM polynucleotide concentration (expressed in terms of nucleotide phosphate).

Binding Stoichiometry: Job Plot

Continuous variation method of Job [31, 32] was employed to determine the stoichiometry of binding in each case from fluorescence spectroscopy. At constant temperature, the fluorescence signal was recorded for solutions where the concentrations of both the DNA and the dye were varied while their sum was kept constant. The difference in fluorescence intensity (ΔF) at 615 nm of the dye in the absence and presence of the various DNA conformations was plotted as a function of the input mole fraction of the dye. The stoichiometry was obtained in terms of DNA—dye $\left[(1 - \chi_{dye})/\chi_{dye}\right]$ where χ_{dye} denotes mole fraction of the dye which is the break point in the plot. The results reported are average of at least three experiments.

Circular Dichroic Spectra

Circular dichroic (CD) spectra were measured in a rectangular quartz cell of 1 cm path length. Each spectrum was averaged from four successive accumulations at a scan rate of 100 nm/ min, keeping a bandwidth of 1.0 nm at a sensitivity of 100 millidegree, was baseline corrected and smoothed within permissible limits using the inbuilt software of the unit and normalized to nucleotide concentration in the region of intrinsic CD of the DNA. The molar ellipticity (θ) is expressed in deg. cm^2 $dmol^{-1}$.

Isothermal Titration Calorimetry

For isothermal titration calorimetry (ITC) experiments aliquots of degassed DNA solution was titrated by injecting from a rotating syringe (290 rpm) into the isothermal sample chamber containing the dye solution (1.4235 mL). Corresponding control experiments to determine the heat of dilution of DNA were performed by injecting identical volumes of same concentration of DNA into buffer. The area under each heat burst curve was determined by integration using the Origin 7.0 software to give the measure of the heat associated with the injection. The heat associated with each DNA-buffer mixing was subtracted from the corresponding heat of DNA–dye reaction to give the heat of dye-DNA binding. The heat of dilution of injecting the buffer into the dye solution alone was observed to be negligible. The resulting corrected injection heats were plotted as a function of the P/D molar ratio and fit with a model for one set of binding sites and analyzed using Origin 7.0 software to provide the binding affinity (K_a), the binding stoichiometry (N) and the enthalpy of binding (ΔH). The binding Gibbs' energy (ΔG) and the entropic contribution to the binding ($T\Delta S$) were subsequently calculated from standard relationships described previously [23, 33]

Results and Discussion

Absorption Spectral Studies

The visible absorption spectrum of thionine has one peak in the 450–700 nm region that is useful for characterizing its interaction with DNA. In Fig. 2, the comparative absorption titration data of a constant concentration of thionine with increasing concentrations of ds and ss DNAs are presented. Significant features in the spectra are hypochromic effects and bathochromic shifts with a single isosbestic point in both cases. These features are much more pronounced in thionine-ds DNA (Fig. 2a) compared to ss DNA (Fig. 2b) interaction. The hypochromicity change was about 48% with the ds DNA and only about 23% with the ss DNA. The spectral changes were utilized to construct Scatchard plots to quantify the binding reaction. Non-linear Scatchard plots (not shown) were further analyzed by non-cooperative equation of McGhee and von Hippel as reported earlier [16, 17, 20] and the results are presented in Table 1. It was observed that the binding to ds DNA was characterized by a very high binding affinity (K_i) value of $(1.34\pm0.20\times10^5)$ M^{-1} compared to the value of $(0.46\pm0.013\times10^5)$ M^{-1} for ss DNA. Furthermore, the number of excluded binding sites was about 5.6 in ds DNA compared to 7.4 with ss DNA again reflecting a closer binding of the thionine molecules on the ds DNA conformation.

Fluorescence Titration Experiments

Having observed that thionine prefers to bind strongly to ds DNA, we studied the interaction observing the change in fluorescence. Thionine has a strong fluorescence spectrum with maximum around 615 nm when excited at 596 nm [16]. This property was exploited to understand the interaction phenomena with the two DNA conformations. Binding to both DNA conformations resulted in the quenching of the fluorescence intensity to different extents eventually leading to saturation of the binding sites. Comparative fluorescence patterns of the complexation of thionine with the two DNA conformations are presented in Fig. 3. The fluorescence quenching was larger in ds DNA (86%), and only 57% in ss DNA indicative of the stronger association of the dye to the ds regions of the DNA structures. The quenching may be due to an effective overlap of the bound molecules in the hydrophobic environment essentially in an intercalated state with the base pairs. The Scatchard binding isotherms constructed from this data were non-linear plots (inset of Fig. 3). The binding constants for ds and ss DNA calculated from the fluorescence data as per the McGhee-von Hippel analysis employing non-linear equation yielded values of 1.22±

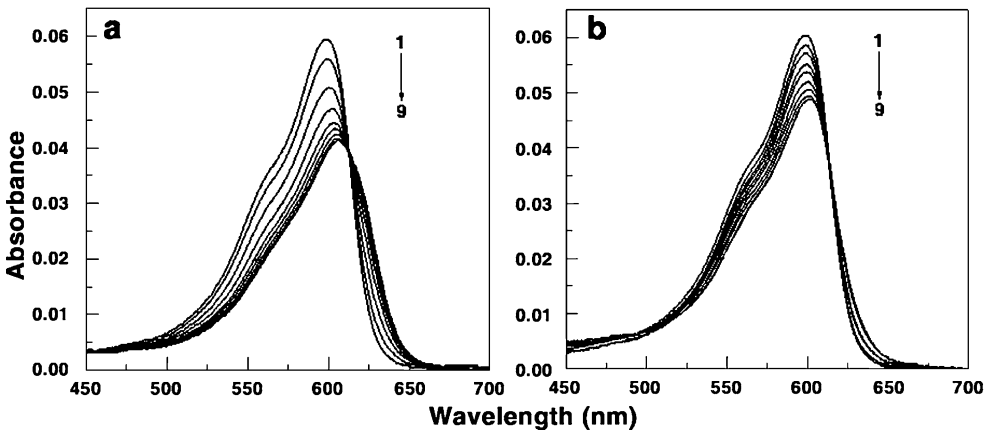

Fig. 2 Representative absorption spectral changes of thionine (1.09 μM) treated with **a** 0, 1.97, 3.94, 7.88, 11.82, 15.76, 19.70, 21.67, 23.64 μM (*curves 1–9*) of ds CT DNA and **b** thionine (1.11 μM) treated with 0, 1.45, 2.90, 4.35, 8.71, 11.61, 21.05, 28.31, 35.57 μM (*curves 1–9*) of ss CT DNA. All experiments were performed in 50 mM sodium cacodylate buffer, pH 7.2 at 20±0.5 °C

Table 1 Binding parameters for the complexation of thionine to ds and ss calf thymus DNA evaluated from Scatchard analysis of the absorbance and fluorescence titration data

DNA conformation	Absorbance		Fluorescence	
	$K_i \times 10^{-5}$ (M^{-1})[a]	*n*	$Ki \times 10^{-5}$(M^{-1})	*n*
Double stranded	1.34±0.20	5.6	1.22±0.20	5.8
Single stranded	0.46±0.013	7.4	0.53±0.30	7.5

Average of four determinations

[a] Binding constants (*Ki*) and the number of binding sites (n) refer to solution conditions of 50 mM cacodylate buffer, pH 7.2 at 20 °C

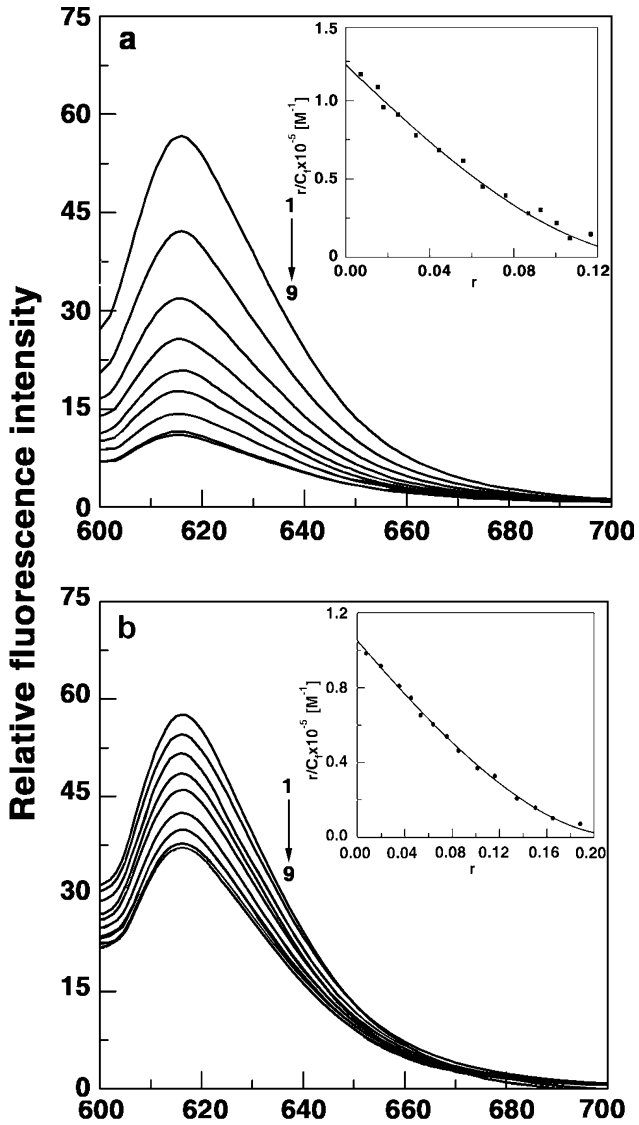

Fig. 3 Representative steady state fluorescence emission spectral changes of thionine (1.05 μM) treated with **a** 0, 3.87, 7.74, 15.48, 19.35, 23.22, 30.96, 38.70, 42.50 μM (*curves 1–9*) of ds CT DNA and **b** 0, 7.26, 10.89, 18.15, 25.41, 39.93, 54.45, 68.97, 76.23 μM (*curves 1–9*) of ss CT DNA. All experiments were done in 50 mM sodium cacodylate buffer of pH 7.2 at 20±0.5 °C. Inset: representative Scatchard plots of the binding

0.20×10^5, and $0.53 \pm 0.30 \times 10^5$ M^{-1}, respectively, for ds and ss DNAs (Table 1). Thus, the fluorescence data also clearly suggest that thionine binds strongly to ds DNA and weakly to ss DNA. The number of occluded binding sites was found to be 5.8 for ds and around 7.5 for ss DNA.

Competition Dialysis Results

The result from the competition dialysis assay in terms of accumulation of the dye in the dialysis tubing was higher with ds DNA (25.75 μM). Very low accumulation was observed with ss DNA (9.62 μM). The striking result that emerged from this experiment was the pronounced binding to ds DNA compared to ss DNA in excellent agreement with the results form spectroscopy. From the concentrations of bound dye, the apparent binding affinities (K_{app}) for the binding of thionine to the two DNA conformations have been calculated and these values were 1.32×10^5 M^{-1} and 0.44×10^5 M^{-1}, respectively. These results clearly indicate that the binding affinities of thionine was remarkably higher with ds DNA compared to ss DNA.

Binding Stoichiometry: Job Plot Results

To establish the binding stoichiometry of thionine with the two DNA conformations, continuous variation analysis procedure (Job plot) was performed in fluorescence. The plots (not shown) of the difference fluorescence intensity (615 nm) versus dye mole fraction revealed single binding event in each case. The intersection of least square fitted lines at χ=0.143 and 0.106 with ds and ss DNA structures corresponds to site size values of 5.96 and 8.36, respectively, for binding to ds and ss DNAs close to the values obtained from Scatchard plots derived from spectrophotometric and fluorimetric titration data (*vide supra*).

Fluorescence Quenching Studies by Ferrocyanide Ions

The mode of binding of thionine to ds DNA was proposed to be intercalative from various experiments previously [7, 14]. Here we probed the comparative ability to intercalate to ds and ss DNA using fluorescence quenching experiments

using the anionic quencher $[Fe(CN)_6]^{4-}$. An anionic quencher would not be able to penetrate the negatively charged barrier around the double helix and for bound molecules buried within the ds DNA helix by intercalation little or no change in its fluorescence is expected. Thus quenching studies are straight forward experiments and provide indication about the location of the bound molecules to be either on the surface or inside of the helix. Stern–Volmer plots for the quenching of thionine fluorescence with the three DNA helical structures are shown in Fig. 4. Results reveal that free molecules are quenched efficiently by the $[Fe(CN)_6]^{4-}$. Very little quenching was observed with molecules complexed with ds DNA while profuse quenching close to that of free molecules was found for molecules bound to ss DNA. This result indicates that the dye molecules bound in the ds DNA are located in a relatively more protected environment, presumably more deeply and/or strongly intercalated compared to that in the ss DNA. It is likely that molecules bound to ss DNA are quenched efficiently like that of the free molecules as they are easily accessible to the quencher. The Stern–Volmer quenching constants calculated for thionine complexes with the ds and ss DNAs were 25.92 and 40.33 M^{-1}, respectively (Table 2). From these results it can be clearly inferred that the dye molecules bound to ds DNA is more protected and are sequestered away from the solvent indicating strong intercalative binding. Those molecules bound to ss DNA are accessible to the quencher to the maximum extent and the binding appears to be on the periphery of the helix as intercalation is not feasible. This experiment clearly proves that thionine binds to ds DNA by intercalation in confirmation to our earlier results [16, 17].

Fluorescence Polarization

Fluorescence polarization measurement is another reliable technique that can provide information on the mode of binding of small molecules with DNA [34]. Small molecules weakly polarize due to their rapid tumbling motion in aqueous media. But on intercalation their rotational motion will be restricted and the fluorescence polarization would be enhanced. It has been found that the fluorescence polarization of thionine complexed to ds and ss DNAs were 0.186 and 0.071 against a value of 0.039 for the free dye. These are presented in Table 2.

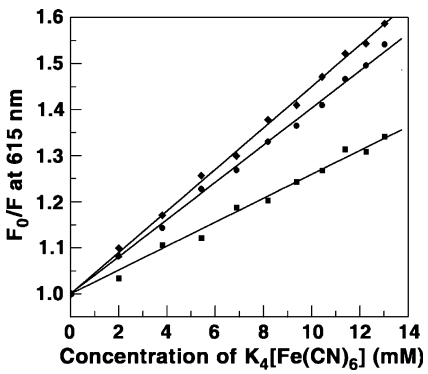

Fig. 4 Stern–Volmer plots for the quenching of thionine (♦) and complexes of thionine-ds CT DNA(■) and thionine-ss CT DNA (●) in 50 mM sodium cacodylate buffer at pH 7.2

Table 2 Various binding parameters for the complexation of thionine to ds and ss calf thymus DNA evaluated from the fluorescence data

DNA conformation	K_{sv} $(M^{-1})^a$	A	$Q_\lambda/Q310$
No DNA	45.00	0.039	–
Double stranded	25.92	0.186	1.32
Single stranded	40.33	0.071	1.08

Average of four determinations

[a] Stern–Volmer constants (K_{sv}) refer to solution conditions of 50 mM cacodylate buffer, pH 7.2 at 20 °C. A is the fluorescence anisotropy

Fluorescence Energy Transfer

The specificity of the binding of thionine to the ds and ss DNA conformations was also verified from contact fluorescence energy transfer. When small molecules intercalate into double stranded regions of DNA, the base pairs can efficiently transfer energy to the excited fluorophore due to their favourable close contacts and the orientation of the donor-acceptor dipoles. Fluorescence energy transfer from nucleotide base pairs to bound dyes is manifested by an increase in the fluorescence quantum yield of bound molecules in the wavelength range corresponding to DNA absorbance [26, 27]. This can be used as an additional evidence for intercalative binding since energy transfer can occur efficiently only if the bound molecule is in close contact with, and oriented parallel to, the base pairs. Figure 5 show plots of the ratio Q_λ/Q_{310} against wavelength

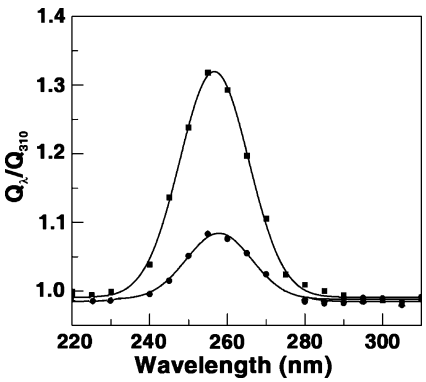

Fig. 5 Variation of relative fluorescence quantum yield of thionine in the presence of ds CT DNA(■) and ss CT DNA (●) in 50 mM sodium cacodylate buffer of pH 7.2 at 20±0.5 °C as a function of excitation wavelength

at various P/D ratios showing an increase in quantum yield in the region of DNA absorbance. The increase in quantum yield as given by Q_λ/Q_{310} was much higher (1.32) for the complexes with ds conformation in comparison to ss DNA conformation (1.08). This indicates that binding to the ds form resulted in substantially higher energy transfer to thionine molecules in the complex compared to ss complexes providing strong evidence for a true intercalative binding to the double helical state.

Salt Dependence Studies

Thionine is a cationic dye. Its binding to DNA has been shown to be dependent on the salt concentration of the medium [16, 35]. To ascertain the extent of electrostatic interaction in its binding to ds and ss DNAs salt dependent fluorescence titrations were performed at three [Na^+] ion conditions viz. 10, 20 and 50 mM. The binding affinity to ds DNA varied from 3.41×10^5 M^{-1} at 10 mM to 1.39×10^5 M^{-1} at 50 mM while that of ss DNA varied only from 1.12×10^5 M^{-1} to 0.41×10^5 M^{-1} in the same range of [Na^+]. The change is very high with the ds DNA and small with ss DNA as the salt is increased implying that the intercalative binding in ds DNA is affected more compared to the binding in ss DNA.

Spectroscopic Study Using Circular Dichroism

The circular dichroic spectra of the different DNA helical forms displayed a gross B-form conformation characterized by a large positive band in the 270–280 nm and a negative band around 245 nm. There are, however, significant differences in the ellipticity and wavelength maxima in each case. The CD bands of the ds form are caused due to the stacking interactions between the bases and the helical structure that provide asymmetric environment for the bases. Thionine is CD inactive molecule. Conformational changes associated in the DNA on the binding of thionine were investigated from circular dichroic studies. To record dye induced conformational changes, the CD spectra in the 210–700 nm regions were recorded in presence of varying D/P (dye/nucleotide phosphate molar ratio) values. In presence of thionine, the ellipticity of the long wavelength positive band of the two DNA conformations increased. The ellipticity changes were high with ds structures and lowest with the ss conformation. The CD spectral data are presented in Fig. 6. Furthermore, a conservative pair of strong induced CD bands appeared on either side of the wavelength maximum of absorption of thionine (598 nm) with both DNA conformations with the positive wavelength maximum around 630 nm and minimum around 313 nm. This may be attributed to an exciton splitting mechanism arising due to the effective

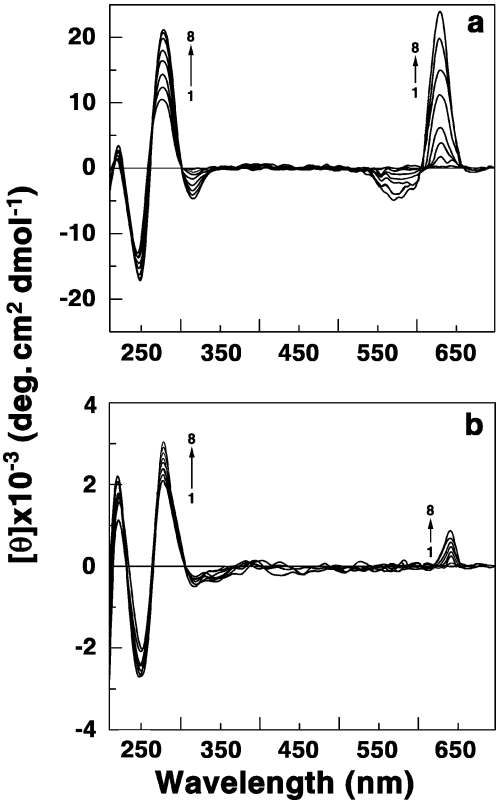

Fig. 6 Representative intrinsic circular dichroic spectral changes of **a** 60 μM ds CT DNA treated with 0, 3, 6, 9, 12, 18, 24, 30 μM of thionine (*curves 1–8*) and **b** 60 μM ss CT DNA treated with 0, 3, 9, 15, 30, 36, 42, 48 μM of thionine (*curves 1–8*). All experiments were performed at 20±0.5 °C in 50 mM sodium cacodylate buffer at pH 7.2

interaction of the transition moments of intercalated dye molecules with that of the base pairs whereby the isohelical arrangement of the drug molecules on the DNA results in an asymmetric arrangement of the dye chromophores. The positive induced band ellipticity for thionine-ds DNA conformation (Fig. 6a) was 20,600 deg cm^2 $dmol^{-1}$ while for ss DNA (Fig. 6b) it was only 3,100 deg cm^2 $dmol^{-1}$. The negative band ellipticity values for ds and ss DNAs were 23,800 and 950 deg cm^2 $dmol^{-1}$, respectively. The induced CD band ellipticity thus was very high with ds DNA and the large differences in the intensities indicate that the binding is sensitive to the duplex nature, being very small with single stranded DNA conformation where intercalation is not feasible. Thus, the magnitude of the induced CD spectral bands may suggest differences in the strength of the bound molecule inside the helical organization of the DNA conformations.

Isothermal Titration Calorimetric Studies

To complement the structural data, we studied the thermodynamics of the interaction of thionine with the

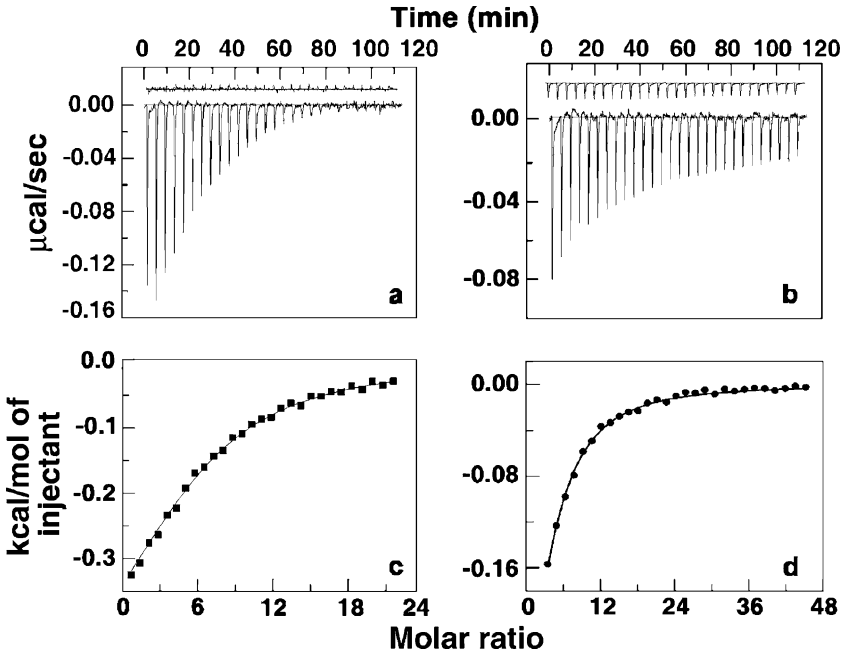

Fig. 7 Representative ITC profile for the titration of **a** ds CT DNA and **b** ss CT DNA with thionine at 20 °C in 50 mM sodium cacodylate buffer of pH 7.2. The *top panels* represent the raw data for the sequential injection of DNA into thionine and the *bottom panels* show the integrated heat data after correction of heat of dilution against the molar ratio of DNA/drug. The data points (■, thionine- ds CT DNA; ●, thionine-ss CT DNA) reflect the experimental injection heat, which were fitted to one site model, and the solid lines represent the best-fit data

ds and ss helical conformations of DNA to understand the energetics involved in the binding. The isothermal titration calorimetric patterns are presented in Fig. 7. In both cases the binding had only single event. ds DNA binding generated more heat compared to ss DNA. The thermodynamic parameters elucidated from the titration are presented in Table 3. The binding affinity (K_a) for ds DNA was 1.39×10^5 M^{-1} and with the ss DNA it was 0.45×10^5 M^{-1} in good agreement with that obtained from spectroscopic experiments. The site size (n) values were about 5.9 and 7.2 nucleotides. The overall binding affinity and the binding site size values obtained from ITC analysis are in excellent agreement with the affinity values and the stoichiometry values from spectroscopy. It can be seen that Gibbs' energy for the binding was higher for ds compared to that for ss DNA. Binding to ds DNA was favored by negative enthalpy and positive entropy contributions while the binding to ss DNA was predominantly entropy favoured with a smaller enthalpy term. Intercalative binding of small molecules are usually both enthalpy and entropy favoured [23, 35] as observed here.

Conclusions

This study presents the structural and energetic aspects of the binding of the phenothiazinium dye thionine with double and single stranded helical conformations of CT DNA using absorption, fluorescence, competition dialysis, circular dichroic and calorimetric techniques. The conclusions from the data obtained from absorbance and fluorescence techniques suggest the high preference of thionine to ds conformation over ss structure. The binding also resulted in induction of strong optical activity in the bound dye molecules and the same was stronger with ds conformation. Thermodynamics of the interaction suggests enthalpy and entropy favored binding to ds DNA. The results unequivocally present the high preference of thionine to ds

Table 3 Thermodynamic parameters for the association of thionine with double and single stranded DNA from isothermal titration calorimetry

DNA conformation	$K_a\times10^{-5}$ (M^{-1})	n	ΔG (kcal /mol)	ΔH (kcal /mol)	$T\Delta S$ (kcal/mol)
Double stranded	1.39	5.98	−6.91	−3.39	3.52
Single stranded	0.45	7.24	−6.25	−1.12	5.13

All the data in this table are derived from ITC experiments conducted in cacodylate buffer of 50 mM [Na^+], pH 7.2 and are average of four determinations. K_a and ΔH values were determined from ITC profiles fitting to Origin 7.0 software as described in the text. The values of ΔG were determined using the equation $\Delta G=\Delta H-T\Delta S$. n is the reciprocal of N, the stoichiometry. All the ITC profiles were fit to a model of single binding site

conformation and prove the strength of spectroscopic techniques to clearly elucidate the binding aspects, preferences and mode of interaction.

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