Interaction of ct-DNA with 2,4-Dihydroxy Salophen

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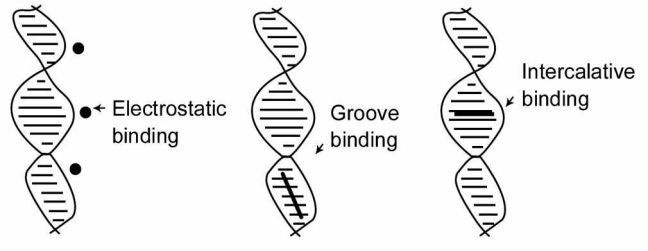
In the present study, at first, 2,4-Dihydroxy Salophen (2,4-DHS), has been synthesized by combination of 1, 2-diaminobenzene and 2,4-dihydroxybenzaldehyde in a solvent system. This ligand containing *meta*-quinone functional groups were characterized using UV-Vis and IR spectroscopies. Subsequently, the interaction between native calf thymus deoxyribonucleic acid (ct-DNA) and 2,4-DHS, was investigated in 10 mM Tris/HCl buffer solution, pH 7.2, using UV-visible absorption and fluorescence spectroscopies, thermal denaturation technique and viscosity measurements. From spectrophotometric titration experiments, the binding constant of 2,4-DHS with ct-DNA was found to be $(1.1 \pm 0.2) \times 10^4$ M^{-1}. The fluorescence study represents the quenching effect of 2,4-DHS on bound ethidium bromide to DNA. The quenching process obeys linear Stern-Volmer equation in extended range of 2,4-DHS concentration. Thermal denaturation experiments represent the increasing of melting temperature of DNA (about 3.5 $^{\circ}C$) due to binding of 2,4-DHS. These results are consistent with a binding mode dominated by interactions with the groove of ct-DNA.

Key Words: DNA, Schiff base, Interaction, Fluorescence, UV-Vis

Introduction

Schiff bases, analogously to porphyrins, shows a catalytic activity toward the bland oxidation of hydrocarbons and undergoes electron transfer reactions, mimicking the catalytic functions of peroxidases.[1,2] Moreover, the affinity of some Schiff bases for the oxygen molecule and the capability to form oxo-complexes is similar to what observed for some porphyrins derivatives.[1,2] The study of the interaction of Schiff bases with DNA has been the focus of some recent research works.[3] This is partly because that, Chemical carcinogens, radiation and many chemical antitumor agents share a common property in that they exert their biological effects through mechanisms involving DNA damage. On the one hand, certain DNA-bound forms of chemotherapeutic agents are responsible for arresting tumor cell growth. On the other hand, some of the damages induced by carcinogenic chemicals are the mediators of mutational change, which, in turn, are likely to be necessary prerequisites for carcinogenic transformation. The studies of the molecular details of these binding interactions have been crucial in rational design of new antitumor agents as well as developing sensitive probes of local nucleic acid structure.

Three major binding modes have been proposed for the binding of Schiff bases to DNA: intercalation, outside groove binding and outside binding with self-stacking in which the Schiff bases are stacked along the DNA helix (Scheme 1). The interaction of multidentate aromatic ligands, with DNA has recently gained much attention following the important biological and medical roles played by potential these intercalators.[3,4] The interaction of H_2Salen transition metal complexes, including Cu,[5,6] Ni,[5,7-9] Mn,[5,10,11] Co[5,12] and Ru,[13] with DNA was studied and great changes of spectroscopic properties were generally noticed, indicating binding interactions between DNA and such compounds. An intercalating interaction mode was proposed for Co (Salen),[5,12] an external binding with the surface of the double helix was suggested for a functionalized Cu (Salen) complex,[5,6] while Ni (Salen) presented a high affinity toward the N_7 atom of guanosine residues.[5,7-9] On basis of our knowledge, no work has been published on the interaction of 2,4-Dihydroxy Salophen (2,4-DHS) with DNA. In this work, we present a comprehensive study on the interaction between DNA and this new bifunctional Schiff base molecule containing both aromatic domains and electroactive functional groups. This new molecule can be easily synthesized in high yield using inexpensive starting materials. The mode of interaction with DNA has been elucidated by spectroscopic techniques. Experimental results suggest that this molecule is capable of binding specifically to ct-DNA sequences.



Scheme 1. Three major binding modes for the binding of Schiff bases to DNA: intercalation, outside groove binding and outside binding

Experimental

Chemicals and solutions. 1,2-Diaminobenzene and 2,4-dihydroxybenzaldehyde, were obtained from Aldrich Chemical Co. and were used as received. Anhydrous dimethylformamide (DMF) was purchased from Sigma. All other chemicals used in this work, were reagent quality, obtained from Sigma-Aldrich Co. and used as received without further purification. 2,4-DHS has been synthesized by combination of 1,2-diaminobenzene

and the 2,4-dihydroxy benzaldehyde. This ligand containing meta-quinone functional groups was characterized using UV-Vis and IR spectroscopies, in non aqueous solvents, such as dimethylformamide (DMF). Stock solution of 2,4-DHS (typically 10 mM) was prepared just prior to use by dissolving the solid in DMF. Water was purified with a Millipore Milli-Q system and all the experiments were carried out at room temperature. Double stranded calf thymus DNA (ct-DNA, activated and lyophilized) was purchased from Sigma. ct-DNA stock solutions (2 mg/mL) were prepared in 10 mM Tris/HCl, pH 7.2 buffer. The DNA solutions gave a UV absorbance ratio (A_{260}/A_{280}) of about 1.9, indicating that the DNA was sufficiently free from protein.[14] The concentration in base pairs of DNA was determined using an extinction coefficient of 6600 $cm^{-1}M^{-1}$ at 260 nm.[15]

Synthesis of 2,4-Dihydroxy Salophen. According to the traditional procedure of synthesis of tetradentate Schiff base ligands,[16] the reaction of salicylaldelhydes with 0.5 mol equivalent of diamines, in refluxing MeOH for a few hours, gives rise to the final products (75 ~ 85% yield) which were analytically pure solids after recrystalization. In a 25 mL rounded bottom flask 1,2-diaminobenzene (0.1080 g; 1.00 mmol) was mixed with 2,4-dihydroxybenzaldehyde (0.2762 g; 2.00 mmol) then added 25 mL ethanol 95% and refluxed. Afterwards the mixture was refluxed for 3 h a fine yellow solid mixture was obtained. The obtained precipitate was filtered and washed several times with ethanol 96% and ether. The product was recrystalized by ethanol to obtain yellow needles of pure 2,4-DHS (0.2819 g, 81%). The chemical structure of 2,4-DHS was shown in Scheme 2.

Spectroscopy. The IR measurements were done on Philips spectrophotometer. The absorbance measurements were carried out using UV-Vis. Carry-500 double beam spectrophotometer, operating from 200 to 800 nm in 1.0 cm quartz cells. The absorbance titrations were performed at a fixed concentration of the specific 2,4-DHS ligand and varying the concentration of double stranded ct-DNA. In order to prevent interferences due to DNA absorption, the data were obtained by keeping the same concentration of ct-DNA in the reference cuvette. All the curves presented were baseline-corrected using the application included in Carry Win UV Version 4.9 software.

Fluorescence spectra were recorded with a RF-5000 Shimadzu spectrofluoremeter equipped with a Peltier system to control the temperature inside the cuvettes. All the curves presented were baseline-corrected using the application included in RF-5000 digital station software.

To allow their equilibration, samples of aqueous 2,4-DHS solutions were let for one week at room temperature. After this equilibration, stable and reproducible UV-Vis absorption spectra were obtained. All the solutions were extensively degassed under vacuum before measurements.

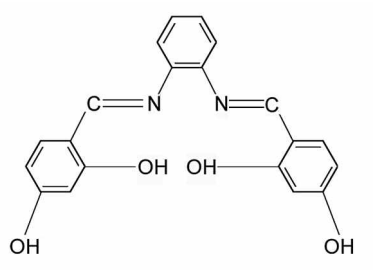
Melting experiments. Melting curves were performed using an UV-Vis Carry-500 double beam spectrophotometer in conjunction with a thermostated cell compartment. The measurements were carried out in 10 mM Tris/HCl, pH 7.2. The temperature inside the cuvette was determined with a platinum probe and was increased over the range 20 ~ 90 °C at a heating rate of 1 °C/min (Thermal sortware). The melting temperature, T_m, was obtained from the mid-point of the hyperchromic transition.

In all of the experiments, for the pH measurement, we used a potentiometer (Metrohm model, 744).

Viscosity measurements. The viscosity of ct-DNA solutions was measured at 25 ± 0.1 °C using an Ubbelohde viscometer. Typically, 10 mM Tris/HCl buffer solution, pH 7.2 was transferred to the viscometer to obtain the reading of flow time. For determination of solution viscosity, 10.0 mL of buffered solution of 160 μM ct-DNA was taken to the viscometer and a flow time reading was obtained. An appropriate amount of Schiff base was then added to the viscometer to give a certain R (R = [Schiff base]/[DNA base pair]) value while keeping the ct-DNA concentration constant, and the flow time was read. The flow times of samples were measured after the achievement of thermal equilibrium (30 min). Each point measured was the average of at least five readings. The obtained data were presented as relative viscosity, η/η_o, versus R, where η is the reduced specific viscosity of DNA in the presence of Schiff base and η_o is the reduced specific viscosity of ct-DNA alone.[17,18]

Results and Discussion

Structural characterization of 2,4-DHS. IR spectra of tetradentate 2,4-DHS ligand, synthesized according with the procedures described in Section 2, had this IR information (KBr): $\nu_{C=N}$ 1614 cm^{-1}; $\nu_{Ar\text{-}O\text{-}H}$ 1300 cm^{-1}; $\nu_{C=C}$ 1486 cm^{-1}; $\nu_{Ar\text{-}H}$ 730 cm^{-1}. The IR spectrum was shown in Fig. 1. The significant frequencies were selected by comparing the IR spectra of the purified ligands with those obtained for the commercially available salophen. This Schiff base ligand present a characteristic band in the range of 1605 ~ 1630 cm^{-1} attributable to the stretching vibration of the azomethine (C = N) group. The presence of



Scheme 2. The chemical structure of 2,4-DHS

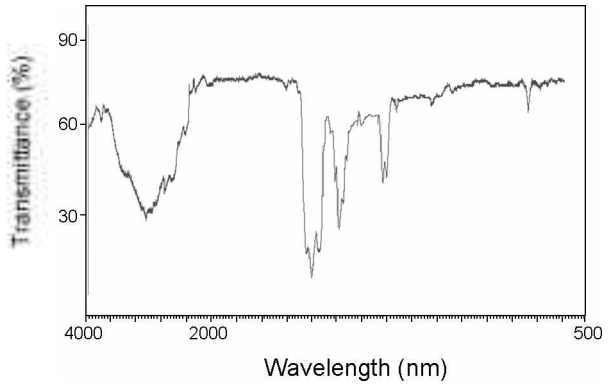


Figure 1. The IR spectra of 2,4-DHS.

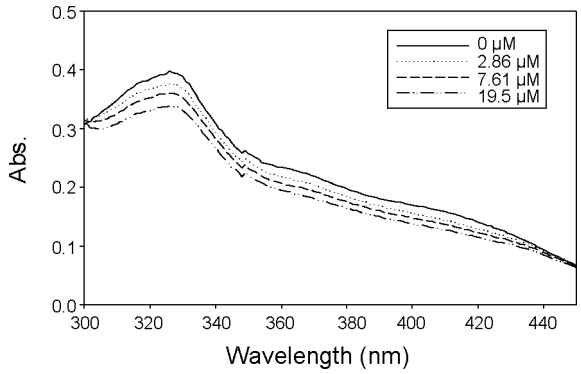


Figure 2. The spectrum of 2,4-DHS (14.2 μM) in the presence of 0, 2.86, 7.61, 19.5 μM of ct-DNA. Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C.

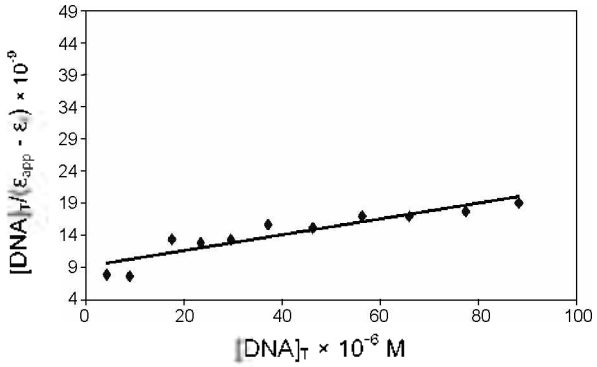


Figure 3. The plot of $[DNA]_T/(|\varepsilon_{app} - \varepsilon_f|)$ versus $[DNA]_T$

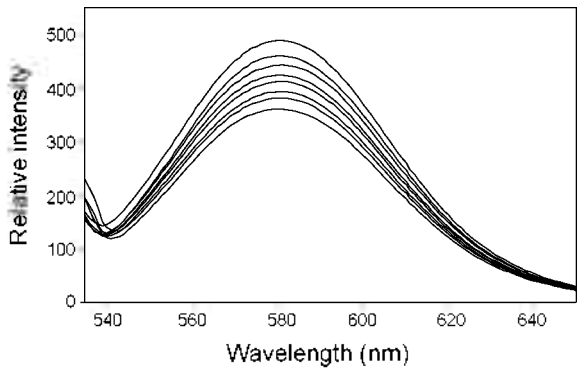


Figure 4. The emission spectrum of EB (5 μM) bound to DNA (12 μM) in the presence of various amounts of 2,4-DHS. Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C. The peak area is decreased with increasing of 2,4-DHS concentration (0, 0.31, 0.52, 0.74, 0.85, 0.94, 1.2, 1.32 μM) in the direction of arrow. The excitation wavelength was 515 nm.

this band is concomitant with the absence of absorption bands in the range of 1680 ~ 1690 cm^{-1} corresponding to the starting carboxaldehyde groups. In addition the presence of phenolic hydroxylic groups can be ascertained by the presence of strong absorption bands in the range of 1270 ~ 1290 cm^{-1}. The absorbance spectrum of 2,4-DHS also shows the maximum wave length at 331 nm.

UV-Vis spectral studies. The binding of certain complex to DNA can be produced hypochromism, a broadening of the envelope, and a red shift of the complex absorption band. These effects are particularly pronounced for intercalators, with groove binders, a large wavelength shift usually correlates with a complex conformational change on binding or complex-complex interactions. A spectral change of 2,4-DHS due to addition of DNA was shown in Fig. 2. For obtaining these spectra, the fixed amount of Schiff base in Tris-HCl buffer pH 7.2 was titrated with a stock solution of ct-DNA. The changes in absorbance of the Soret band upon addition of ct-DNA were monitored at the maximum of the Soret band. It exhibited the low hypochromism and negligible red shift due to the incremental addition of ct-DNA indicating groove binding mode. The apparent binding constant, K_{app}, for the interaction between the 2,4-DHS ligand and ct-DNA can be determined by analysis of absorption spectrophotometric titration data at room temperature using Eq. (1):[19]

$$\frac{[DNA]_T}{(|\varepsilon_{app} - \varepsilon_f|)} = \frac{[DNA]_T}{K_{app}(|\varepsilon_b - \varepsilon_f|)} + \frac{1}{(|\varepsilon_b - \varepsilon_f|)} \quad (1)$$

where $[DNA]_T$, ε_{app}, ε_f and ε_b correspond to total concentration of ct-DNA base-pair, A_{obsd}/[Schiff base], the extinction coefficient for the free Schiff base and the extinction coefficient for the Schiff base in the fully bound form, respectively. In the plot of $[DNA]_T/(|\varepsilon_{app} - \varepsilon_f|)$ versus $[DNA]_T$ that was shown in Fig. 3. K_{app} is given by the ratio of the slope to the intercept .The apparent binding constants of 2,4-DHS was calculated to be $(1.1 \pm 0.2) \times 10^4\ M^{-1}$. Small red shift in Soret band (3 nm) and decrease the absorbance intensity in Soret absorbance intensity (7%) are the reasons that show us the groove binding interaction.[20]

Fluorescence spectroscopic studies. Ethidium bromide (EB) emits intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It was previously reported that the enhanced fluorescence can be quenched by the addition of a second molecule.[21,22] In fluorescence titration experiment, the specified volumes of concentrated 2,4-DHS solution were added consecutively to the cell which contain 2000 μL DNA (12 μM) and EB (5 μM) solution. Representative emission spectra of EB-DNA solution in the presence of various amounts of 2,4-DHS were shown in Fig. 4. The pecks area was measured and analyzed using the classical Stern-Volmer equation (Eq. (2)):[23]

$$I_o/I = 1 - KR \quad (2)$$

where I_0 and I are the fluorescence area peck in the absence and the presence of ligand, respectively, K a linear Stern-Volmer quenching constant, R the ratio of total concentration of ligand to that of ct-DNA. The quenching extent of fluorescence of EB bound to ct-DNA is used to determine the extent of binding between the second molecule and ct-DNA. The Stern-Volmer plot for fluorescence quenching of EB bound to ct-DNA by the Schiff base was shown in Fig. 5. The quenching plots illustrate that the quenching of EB bound to ct-DNA by the Schiff base is in good agreement with the linear Stern-Volmer equation, which also proves that the Schiff base bind to DNA. In the plot of I_0/I versus R = [Schiff base]/[DNA], K is given by the ratio

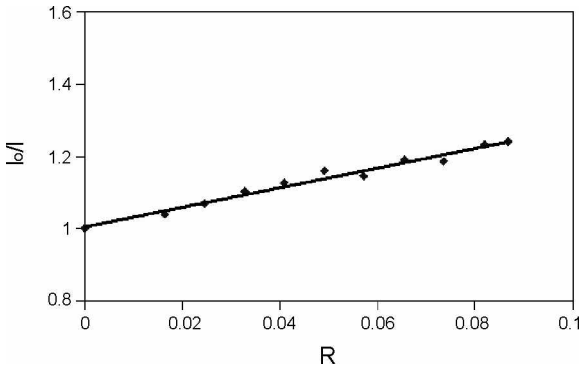


Figure 5. The Stern-Volmer plot for quenching of EB bound to DNA by 2,4-DHS ligand.

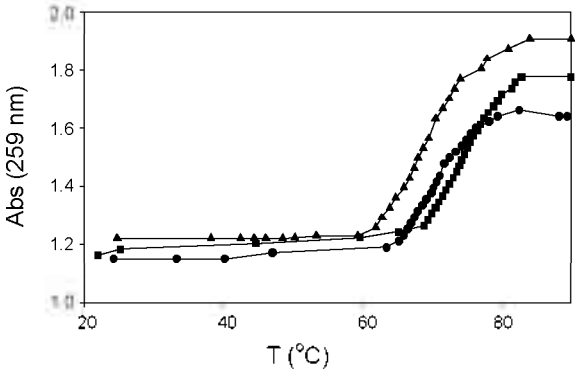


Figure 6. Thermal denaturation curves of DNA in the various [2,4-DHS]/[DNA] molar ratios of R_1 = 0.0 (▲), R_2 = 0.2 (♦) and R_3 = 1.0 (■). Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C.

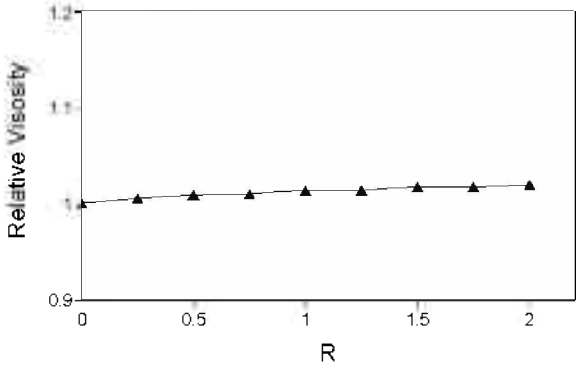


Figure 7. Relative viscosity of calf thymus DNA (7.8×10^{-5} M) in the presence of increasing amounts of 2,4-DHS at stoichiometric ratios R = [2,4-DHS]/[DNAphosphate] = 0.0-1.0, plotted as $(\eta/\eta_0)^{1/3}$ *vs.* R. Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C.

of the slope to intercept. The *K* value for 2,4-DHS was 5.14. The relative value of obtained K respect to other known intercalators represents the non-intercalation mode for 2,4-DHS.[24]

Thermal denaturation experiments. When a molecule intercalates to ct-DNA, the stability of the helix increases and, as a result, the temperature at which the helix denatures (T_m) goes up around 5 ~ 12 °C in R = 1.[25,26] Thus, this parameter is most useful in analyzing the mode of interaction. The melting plot of ct-DNA was monitored by plotting the variation of maximum absorption of ct-DNA solution (100 μM) at 258 nm *vs.* temperature (Fig. 6). The denaturation curves were measured at various R = [2,4-DHS]/[DNA] molar ratios. The melting temperature of ct-DNA (T_m) was estimated from the midpoint of transition curve. With respect to this curve, the melting temperature of ct-DNA has been increased about 3.5 °C in the presence of 2,4-DHS in R = 1. The stabilization of the DNA double helix by binding of the 2,4-DHS increases smoothly with an increasing molar ratio. 2,4-DHS show duplex stabilization. The rings of 2,4-DHS had an important effect and they increased DNA affinity.

This observing small change in the T_m of ct-DNA in the presence of 2,4-DHS suggests that the interaction of these compounds with ct-DNA does not involve intercalation between the base pairs and could be ascribed to interactions with the DNA grooves.[27]

Viscosity measurements. Optical or photo physical probes generally provide necessary, but not sufficient, clues to support kind of binding model. Hydrodynamic measurements that are sensitive to length increases (i.e. viscosity, sedimentation, rotational diffusion as measured by transient electric diffusion) are the least ambiguous and most critical tests of the binding models in solution. The DNA helix lengthens as the base pairs are separated to accommodate the bound complex for the groove binding of the molecule, leading to low increasing in DNA viscosity. Hydrodynamic methods are thus suitable to detect such changes and, in the absence of crystallographic structural data, provide essential evidence to support the intercalation model. In contrast, partial and/or nonclassical intercalation of complex could bend (or kink) the DNA helix, reduce its effective length and in turn, its viscosity. The effect of 2,4-DHS on the viscosity of DNA is shown in Fig. 7. The relative viscosity of DNA shows small increasing with increase in the concentration of the 2,4-DHS, which is not similar to that of the classical intercalation (i.e. ethidium bromide).[28,29] The viscosity results unambiguously show that 2,4-DHS bind with DNA by groove binding mode, this results are in agreement with optical absorption experiments.

Conclusion

In this work, we present a comprehensive study of the interaction between DNA and a new bifunctional Schiff base molecule containing both aromatic domains and electroactive functional groups. This new molecule can be easily synthesized in high yield using inexpensive starting materials. The mode of interaction with DNA has been elucidated by combining some techniques. The results obtained from fluorescence, UV-Vis, thermal denaturation and viscosity measurements. From spectrophotometric titration experiments, the binding constants of 2,4-DHS with ct-DNA were found to be $(1.1 \pm 0.2) \times 10^4$ M^{-1}. The results show that 2,4-DHS bind to DNA by groove binding mode. Our research should be valuable for seeking and designing new antitumor drugs, as well as for understanding the mode of the Schiff base binding to DNA and helical conformations of nucleic acids.

These results are consistent with a binding mode dominated by interactions with the groove of ct-DNA, analogously to what reported for a number of porphyrazines and metal-porphyrazine complexes interacting with DNA.[24]

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