천연 DNA와 2,4-디히드록시살로펜-염화철(III)과 의 상호작용 연구

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(접수 2010. 5. 10; 수정 2010. 6. 4; 게재확정 2010. 8. 18)

Study of Interaction of Native DNA with Iron(III)-(2,4-Dihydroxysalophen)chloride

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(Received May 10, 2010; Revised June 4, 2010; Accepted August 18, 2010)

요약. 본 연구에서는 염화제1철을 2,4-디히드록시살로펜과 조합하여 2,4-디히드록시살로펜-염화철을 합성하였다. 이 복합체를 자외선-가시광선 분광법, IR 분광법으로 분석하였고, 천연의 송아지 흉선 DNA (ct-DNA)와 2,4-디히드록시살로펜-염화철의 상호작용을 자외선-가시광선 분광법, 형광분광분석법, 열변성 분석법, 점성측정법을 이용하여 조사하였다. 분광학적 적정실험으로 밝힌 2,4-디히드록시살로펜-염화철과 ct-DNA 사이의 결합상수는 $(1.6 \pm 0.2) \times 10^3$ M^{-1}이었다. 형광분광분석을으로 브롬화 에티디움의 DNA 결합이 2,4-디히드록시살로펜-염화철에 의하여 저해되는 것을 관찰하였다. 이 저해효과는 2,4-디히드록시살로펜-염화철의 농도에 따라 선형의 Stern-Volmer 방정식을 따른다. 열변성 실험으로 2,4-디히드록시살로펜-염화철이 DNA의 녹는점을 약 4.3 $^\circ$C 증가시킨다는 것을 관찰하였다. 이 결과들은 2,4-디히드록시살로펜-염화철이 대부분 ct-DNA의 큰고랑과 상호작용한다는 모델을 잘 설명하여 준다.

주제어: DNA, 쉬프염기, 상호작용, 형광, 자외선-가시광선 분광법

ABSTRACT. In this study, iron(III)-2,4-dihydroxysalophen chloride (Fe(2,4-DHSalophen)Cl), has been synthesized by combination of 2,4-dihydroxysalophen (2,4-DHSalophen) with FeCl$_2$ in a solvent system. This complex combination was characterized using UV-vis and IR spectroscopies. Subsequently, the interaction between native calf thymus deoxyribonucleic acid (ct-DNA) and Fe(2,4-DHSalophen)Cl, 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 Fe(2,4-DHSalophen)Cl with ct-DNA was found to be $(1.6 \pm 0.2) \times 10^3$ M^{-1}. The fluorescence study represents the quenching effect of Fe(2,4-DHSalophen)Cl on bound ethidium bromide to DNA. The quenching process obeys linear Stern-Volmer equation in extended range of Fe(2,4-DHSalophen)Cl concentration. Thermal denaturation experiments represent the increasing melting temperature of DNA (about 4.3 $^\circ$C) due to binding of Fe(2,4-DHSalophen)Cl. These results are consistent with a binding mode dominated by interactions with the groove of ct-DNA.

Keywords: DNA, Schiff base, Interaction, Fluorescence, UV-vis spectroscopy

INTRODUCTION

The interaction of transition metal complexes, containing multidentate aromatic ligands, with DNA has recently gained much attention following the important biological and medical roles played by potential metallointercalators.[1] Schiff base metal complexes have been recognized as powerful catalysts in a great number of chemical reactions such as, electrochemical reduction of alkyl halides in aprotic solvents,[2] oxygenation of indols, phenols, flavones, and others.[3]

In addition, some of these complexes are able to bind in a reversible form to molecular oxygen. These complexes have been also investigated as model compounds in the study of the natural oxygen carriers such as, hemoglobin, myoglobin hemocyanin[4] and in the study of the catalytic properties of some cytochromes involved in the biological oxidative reactions. Finally, Schiff base tetradentate complexes of iron have been studied as mimetic models of peroxidases, catalases and superoxide dismutase.[5] In addition to these applications, the synthesis and further structural
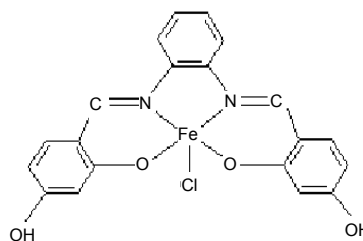
and chemical characterization of new Schiff base tetradentate ligands have been studied in many chemical and biological fields. Three major binding modes have been proposed for the binding of Schiff bases to DNA, intercalation, outside groove binding and outside binding with self-stacking in which the Schiff bases stacked along the DNA helix.[6] The central metal ion strongly influences both the binding characteristics of the Schiff base complex to DNA and the DNA cleavage properties.[7] The interaction of a number of iron(II) and iron(III) derivatives with nucleic acids has been widely investigated, but, no work has been published on the interaction of Fe(2,4-DHSalophen)Cl complexes with DNA. In this study, we have synthesized Fe(2,4-DHSalophen)Cl and investigated its interaction with DNA in aqueous solutions by UV-vis, and fluorescence spectroscopes, thermal denaturation and viscosity measurements.

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-dihydroxybenzaldehyde. This ligand containing meta-quinone functional groups was characterized using UV-vis and IR spectroscopies, in non aqueous solvents, such as dimethylformamide (DMF). Fe(2,4-DHSalophen)Cl, has been synthesized by combination of 2,4-dihydroxy-salophen (2,4-DHSalophen) with $FeCl_2.4H_2O$ in a solvent system. This complex was characterized using UV-vis and IR spectroscopies. Stock solutions of Fe(2,4-DHSalophen) Cl derivatives (typically 10 mM) were prepared just prior to use by dissolving the solids in DMF. All the solutions were extensively degassed under vacuum before measurements.

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.[8] The concentration in base pairs of DNA was determined using an extinction coefficient of 6600 cm^{-1} M-1 at 260 nm.[9]

Scheme 1. The chemical structure of Fe(2 ,4-DHSalophen)Cl

Synthesis of 2,4-DHSalophen ligand

According to the traditional procedure of synthesis of tetradentate Schiff base ligands,[10] the reaction of benzaldehydes 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. To reflux the compounds, 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) and 25 mL ethanol 95%. Afterwards the mixture was refluxed for 3 hours to obtain a fine yellow solid mixture. The obtained precipitate was filtered and washed several times with ethanol 96% and ether. The product was recrystalized by MeOH to obtain yellow needles of pure 2,4-DHS (0.2819 g, 81%).

Synthesis of Fe(2,4-DHSalophen)Cl complex

The complex of Fe(2,4-DHSalophen)Cl *Scheme* 1, was synthesized by combination of 0.174 g (5.0 mmol) sample of 2,4-DHSalophen in 50 mL of ethanol and 0.099 g (5.0 mmol) of $FeCl_2.4H_2O$ in 30ml of ethanol were added to the mixture drop by drop while stirring. Then the mixture was refluxed for 8 hours and cooled to room temperature. The synthesized complex was recrystallized from ethanol by diethyl ether and was filtered and dried under vacuum.

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 Fe(2,4-DHSalophen)Cl complex while varying the concentration of double stranded ct-DNA.

Fluorescence spectra were recorded with a RF-5000 Shimadzu spectrofluorimeter equipped with a Peltier system to control the temperature inside the cuvettes.

To achieve equilibration, samples of aqueous Fe(2,4-DHSalophen)Cl solutions were let for one week at room

temperature. Stable and reproducible UV-vis absorption spectra were obtained at this equilibration. All the solutions were extensively degassed under vacuum before measurements.

Melting experiments

Melting curves were established 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 measured with a platinum probe and was increased over the range 20 - 90 °C at a heating rate of 1 °C/min. 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.[11,12]

RESULTS AND DISCUSSION

Structurals characterization of 2,4-DHS and Fe(2,4-DH-Salophen)Cl

IR spectra of tetradentate 2,4-DHSalophen ligand, synthesized base on the procedures described in Section 2. The IR information of this compound (KBr): $v_{C=N}$ 1614 cm^{-1}; v_{Ar-O-H} 1300 cm^{-1}; $v_{C=C}$ 1486 cm^{-1}; v_{Ar-H} 730 cm^{-1}. The significant frequencies were selected by comparing the IR spectra of the purified ligands with those were 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
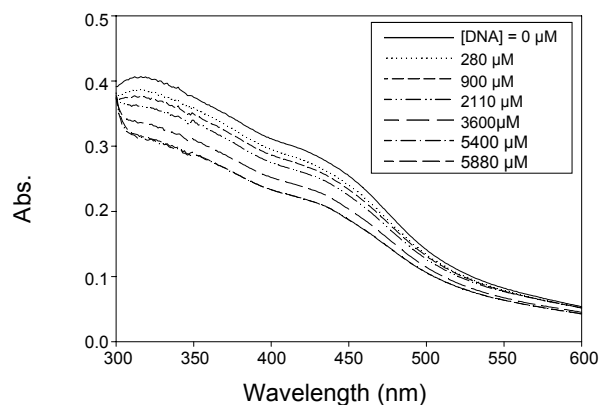

Fig. 1. The spectrum of Fe(2,4-DHsalophen)Cl in the presence of various concentration of ct-DNA. Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C.

(C=N) group. The presence of this band is concomitant with the absence of absorption bands in the range of 1680 - 1690 cm^{-1} corresponding to the starting carboxaldehyde groups. 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-DH-Salophen showed the maximum wave length at 331 nm.[13]

IR spectra of tetra dentate Fe(2,4-DHSalophen)Cl complex, synthesized according with the procedures described in Section 3, contained this IR information (KBr): $v_{C=N}$ 1605 cm^{-1}; v_{Ar-O-H} 1270 cm^{-1}; $v_{C=C}$ 1440 cm^{-1} and v_{Ar-H} 720 cm^{-1}. The decreasing of $v_{C=N}$ in Fe(2,4-DHSalophen)Cl (1605 cm^{-1}) compared to 2,4-DHS (1614 cm^{-1}) is a valuable sign for formation of Fe complex. The absorbance spectrum of Fe(2,4-DHSalophen)Cl also shows the maximum wave length at 316 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 small changes observed on Soret band. A spectral change of Fe(2,4-DHSalophen)Cl due to addition of DNA is shown in *Fig.* 1. For obtaining these spectra, the fixed amount of Schiff base in Tric-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 Fe (2,4-DHsalophen)Cl complex and ct-DNA
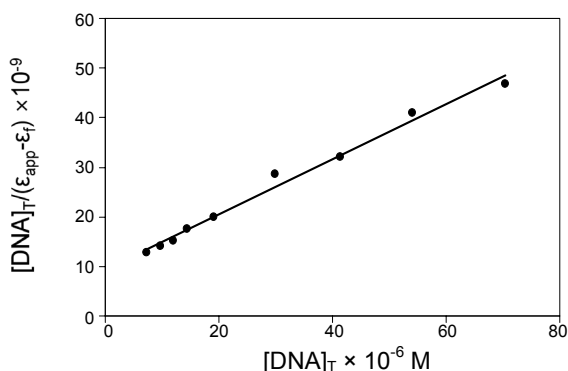
Fig. 2. The plot of $[DNA]_T/(|\varepsilon_{app} - \varepsilon_f|)$ versus $[DNA]_T$.
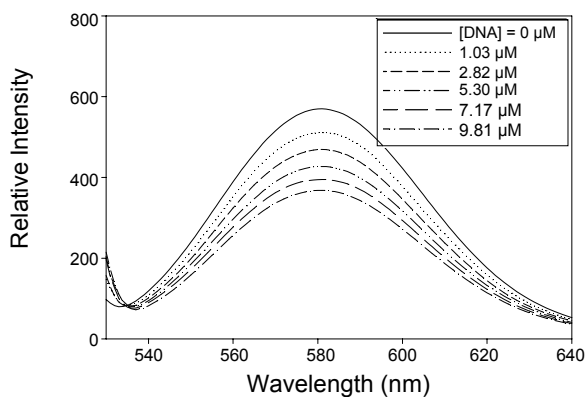

Fig. 3. The emission spectrum of EB (5 μM) bound to DNA (12 μM) in the presence of various amounts of Fe(2,4-DHsalophen)Cl. Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 ºC. The peak area is decreased with increasing of Fe(2,4-DHsalophen)Cl concentration in the direction of arrow. The excitation wavelength was 515 nm.

can be determined by analysis of absorption spectrophotometric titration data at room temperature using Eq. (1):[14]

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

Where $[DNA]_T, \varepsilon_{app}, \varepsilon_f$ and ε_b correspond to total concentration of ct-DNA, $A_{obsd}/[Schiff base]$, 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 is shown in *Fig*. 2, K_{app} is given by the ratio of the slope to the intercept. The apparent binding constant of Fe(2,4-DHSalophen)Cl was determined by $(1.6 \pm 0.2) \times 10^3$ M^{-1}.
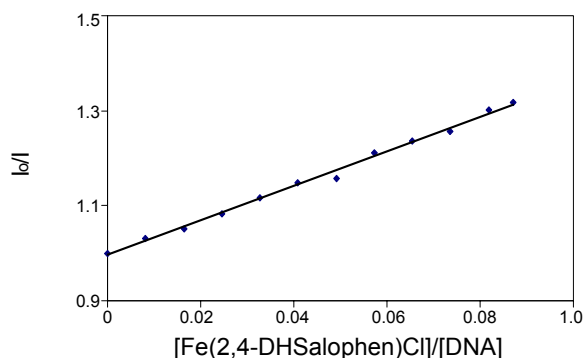

Fig. 4. The Stern-Volmer plot for quenching of EB bound to DNA by Fe(2,4-DHsalophen)Cl complex.

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. As it has been reported, the enhanced fluorescence can be quenched by the addition of a second molecule.[15,16] In fluorescence titration experiment, the specified volumes of concentrated Fe(2,4-DHSalophen)Cl solution were added consecutively to the cell which contained 2000 μL DNA (12 μM) and EB (5 μM) solution. Representative emission spectra of EB-DNA solution in the presence of various amounts of Fe(2,4-DHSalophen)Cl is shown in *Fig*. 3.

The pecks area was measured and analyzed using the classical Stern-Volmer equation Eq. (2):[17]

$$I_o/I = 1 + Kr \quad (2)$$

Where I_0 and I are the fluorescence area peck in the absence and the presence of complex, respectively, K is a linear Stern-Volmer quenching constant and r is the ratio of total concentration of complex to that of ct-DNA. The quenching extent of fluorescence of EB bound to ct-DNA was 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 is shown in *Fig*. 4. 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 confirms the Schiff base bind to DNA. In the plot of I_0/I versus [Schiff base]/[DNA], K is calculated by the ratio of the slope to intercept. The K value for Fe(2,4-DHSalophen)Cl was 6.2 The relative value of K compared with other known intercalators represents the non-intercalation mode for Fe(2,4-DHsalophen)Cl.[13,18]
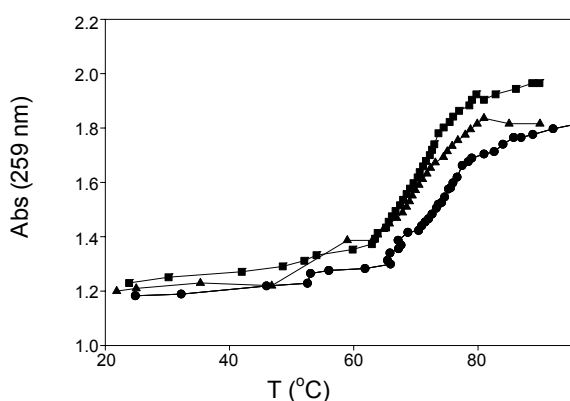
Fig. 5. Thermal denaturation curves of DNA (10.0×10^{-5} M) in the various [Fe(2,4-DHsalophen)Cl]/[DNA] molar ratios of $r_1 = 0.0$ (■), $r_2 = 0.2$(▲) and $r_3 = 1.0$(●). Measurements were done in 10 mM Tris/HCl buffer, pH 7.2 and at 25 °C.
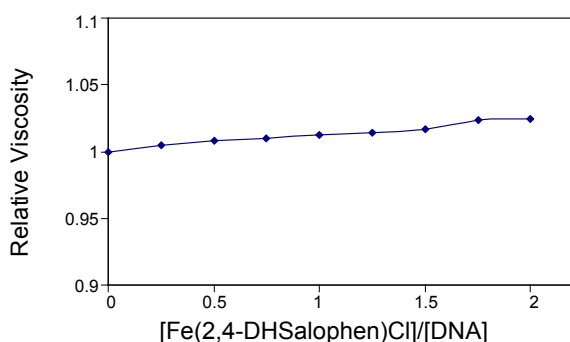

Fig. 6. Relative viscosity of calf thymus DNA (7.8×10^{-5} M) in Tris-HCl 10 mM in the presence of increasing amounts of Fe(2,4-DHSalophen)Cl at stoichiometric ratios r = [Fe(Salen)Cl]/[DNA-phosphate] = 0.0 - 2.0, plotted as $(\eta/\eta_o)^{1/3}$ *vs.* r.

Thermal denaturation experiments

When a molecule intercalates to ct-DNA, the stability of the helix increases and the temperature at which the helix denatures (T_m) goes up varies around 5 - 12 °C in r = 1.[19,20] 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. The denaturation curves were measured at various [Fe(2,4-DHSalophen) Cl]/[DNA] molar ratios and is shown in *Fig.* 5.

The melting temperature of ct-DNA (T_m) was estimated from the midpoint of transition curves. With respecting to the results, the melting temperature of ct-DNA has been increased about 4.3 °C in the presence of Fe (2,4-DHSalophen) Cl. The observed small change in the T_m of ct-DNA in the presence of Fe (2,4-DHSalophen)Cl 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.[21]

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 lower increase 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 Fe(2,4-DHsalophen)Cl on the viscosity of DNA is shown in *Fig.* 6 The relative viscosity of DNA shows small rises with increase in the concentration of the Fe (2,4-DHsalophen)Cl, which is not similar to that of the classical intercalation (i.e. ethidium bromide).[22,23] The viscosity results unambiguously show that Fe(2,4-DHsalophen)Cl bind with DNA by groove binding mode, these results are in agreement with optical absorption experiments.

CONCLUSIONS

The results obtained from fluorescence, UV-vis, thermal denaturation and viscosity measurements exclude DNA outside groove binding. From spectrophotometric titration experiments, the binding constants of Fe(2,4-DHSalophen)Cl with ct-DNA were found to be $(1.6 \pm 0.2) \times 10^3$ M^{-1}. 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 ct-DNA.[18]

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