Electrochemical and Spectroscopic Investigations of Protonated Ferrocene-DNA Intercalation

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The interaction of protonated ferrocene (PF) with chicken blood DNA (CB-DNA) has been investigated *in vitro* by cyclic voltammetry (CV) and UV-Vis spectroscopy as well as viscosity measurements under stomach pH and body temperature. The peak potentials shift in CV, hyperchromism in UV absorption titration, an increase in the viscosity of DNA and the results of the effect of ionic strength on the binding constant strongly support the intercalation of PF into the DNA double helix. The diffusion coefficients of PF in the presence and absence of DNA were 9.54×10^{-11} and 1.34×10^{-10} m²/s, respectively. The binding constant of the PF-DNA complex and the number of binding sites on a DNA molecule were calculated as being 3.07×10^2 M⁻¹ and 2.96, with the help of the Scatchard equation. An expression by Carter *et al.* was used for determining the binding site size (0.17 bp). The binding constant was also determined by UV absorption titration.

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Introduction

Significant progress in molecular biology has resulted in the identification of disorders that are directly related to gene expression, and it is now possible to link some diseases to their responsible specific genes.¹ A number of investigations reveal that the most likely target of antitumor drugs is cellular DNA, and the interaction is such that the tertiary structure of DNA is distorted, thereby inhibiting the replication and transcription machinery of the cell.^{2,3} This damage may lead to various pathological changes in living organisms.⁴ In addition to potential therapeutic applications, due to the redox activity of many drugs, DNA binding studies are anticipated to be useful in electroanalytical applications.⁵⁻⁸

Various techniques have been extensively employed to study the binding of drug molecules to DNA, such as spectroscopic methods,^{9,10} viscometry,¹¹ isothermal calorimetric titration,¹² luminescence,¹³ electrophoresis,¹⁴ fluorescence,¹⁵ nuclear magnetic resonance,¹⁶ quartz crystal microgravimetry¹⁷ and electroanalytical methods.¹⁸⁻²³ Since many small molecules exhibit redox activity, the electrochemical method should provide a useful complement to the previously used methods for investigations.^{18,24-26} Moreover, the electrochemical behaviors are valuable for elucidating some mechanisms of the drug action *in vivo*.^{27,28} Several authors have shown that the binding constants, binding-site sizes and diffusion coefficients of redox-active species can be obtained from straightforward voltammetric experiments in which the DNA is titrated against the redox active species. Measurements of the diffusion currents in the presence of excess nucleic acid have shown that the diffusion coefficient of DNA-bound species is more than an

order of magnitude lower than that of the free species.^{18,29-31}

The interaction of metallo complexes containing multidentate ligands with DNA has recently gained growing interest.^{32,33} In this connection, ferrocene and its derivatives are being paid much attention, and an experimental drug has been reported, which is a ferrocenyl version of tamoxifen.³⁴ The ferrocene molecule is redox active, and it has been suggested that its antineoplastic activity could be connected with its redox processes *in vivo*.³⁵ Hence, the sandwich organometallic compound, ferrocene is a promising candidate for biological applications due to its stability, electrochemical properties and ease of use.^{36,37} The idea is that ferrocene derivatives interact with DNA by intercalation.³⁸⁻⁴⁰ The activity of several anticancer, antimalarial and antibacterial agents and that of aromatic carcinogens find its origin primarily in intercalation.⁴¹⁻⁴³ However, it has also been reported that intercalation is complex, and that the details of the process strongly depend on the structure of the intercalator.⁴⁴ In the present work, the interaction of protonated ferrocene (PF, Scheme 1) with chicken blood DNA (CK-DNA) was investigated in 10% aqueous *N,N*-dimethylformamide (DMF) by cyclic voltammetry (CV),

Scheme 1 Protonated ferrocene.

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UV-Vis spectroscopy and viscometry at stomach pH (4.7) and body temperature (37°C).

Experimental

Reagents and chemicals

Ferrocene (98% purity) was obtained from Fluka, and was used as received. Spectrograde DMF (Sigma-Aldrich, 99.93% purity) was used without further purification. Tetrabutylammonium perchlorate (TBAP) (Fluka, 99% purity) was used as supporting electrolyte, and was further purified by recrystallization using methanol as the solvent. Doubly distilled water was used to prepare all of the solutions, and was buffered at pH 4.7 using 30 mM phosphate buffer *i.e.* Na₂HPO₄ + KH₂PO₄. DNA was extracted from chicken blood by the Falcon method.⁴⁵ A stock solution of DNA was prepared by dissolving an appropriate amount of DNA in doubly distilled water and stored at 4°C. The concentration of a stock solution of CK-DNA (3.54×10^{-4} M in nucleotide phosphate, NP) was determined by UV absorbance at 260 nm using a molar extinction coefficient (ϵ) of $6600 \text{ M}^{-1} \text{ cm}^{-1}$.⁴⁶

A nucleotide-protein ratio (N/P) of 1.75 was determined from UV absorbance at 260 and 280 nm (A_{260}/A_{280}), indicating that the CK-DNA was sufficiently protein free.⁴⁷ A stock solution of PF (6×10^{-3} M) was prepared by dissolving ferrocene in an acidic medium.⁴⁸

Apparatus

Voltammetric experiments were performed using EG and a G Princeton Applied Research (PAR) electrochemical system 370 with a conventional three-electrode cell that consisted of a saturated calomel reference electrode (SCE) from Fisher scientific company (cat No. 13-639-51) in conjunction with an Electrometer Probe Model 178, a thin Pt wire of thickness 5×10^{-4} m with an exposed end of 10^{-2} m as the counter electrode and a bare glassy carbon electrode (GCE) with a geometric area of $7.1 \times 10^{-6} \text{ m}^2$ as the working electrode. Prior to conducting experiments, the GCE was polished with 0.25- μm diamond paste on a nylon buffing pad. For electrochemical measurements the test solution was kept in an electrochemical cell (Model K64 PARC) connected to the circulating thermostat, LAUDA Model K-4R. Absorption spectra were measured on a UV-Vis spectrometer; Shimadzu 1601, and viscosity measurements were made on an Anton Paar Stabinger Viscometer SVM 3000.

Results and Discussion

Electronic spectra

To study the interaction of PF with solution-phase CK-DNA, spectroscopic titrations were carried out at body temperature and stomach pH. While keeping both the concentration and the volume of the PF solution constant, spectroscopic measurements were carried out for monitoring the system while varying the concentration of CK-DNA. The solutions were allowed to equilibrate for 5 min before measurements were made. In the present investigations, the interaction of PF with double-stranded DNA in a mixed solvent of DMF and water was monitored. The absorbance measurements were performed by keeping the concentration of the drug constant (10 μM) in the sample cell, while varying the DNA concentration from 100 to 300 μM in the sample as well as in the reference cell. The spectrum was recorded after each addition of the DNA solution. There was an increase in the absorbance (hyperchromism) as

Fig. 1 Electronic spectra of 10 μM PF in the absence of DNA (a) and the presence of 100 μM (b), 150 μM (c), and 200 μM DNA (d) at pH 4.7 and 37°C.

well as a slight blue shift (2 nm) with the addition of DNA, as shown in Fig. 1.

The observed hyperchromism can be explained by the fact that PF during intercalation undergoes conformational changes in the cyclopentadienyl rings, which could be tilted and rotated like the rings in ferrocene, since the potential barrier to ring rotation is very low (2 – 5 kcal mol⁻¹).⁴⁸ The origin of hyperchromism might lie in the mechanism of the PF interaction with DNA. The introduction of PF in DNA is likely to reduce the face-to-face base stacking, causing an extension of the DNA, which results in the hyperchromic effect. The same effect has also been observed by other investigators for the short bands of certain porphyrins, phenanthroliens and the Al-salophen complex when interacted with DNA.⁴⁹⁻⁵¹ Several authors have suggested that the unstacking of base pairs is the cause of hyperchromism.^{52,53} The binding constant was calculated according to the following equation⁵⁴⁻⁵⁶

$$\frac{A_0}{A - A_0} = \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} + \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} \frac{1}{K_b[\text{DNA}]}, \quad (1)$$

where K_b is the binding constant, A_0 and A are the absorbances of the drug and its complex with DNA respectively, and ϵ_G and ϵ_{H-G} are the absorption coefficients of the drug and the drug-DNA complex, respectively.

The binding constant, K_b ($3.45 \times 10^2 \text{ M}^{-1}$), of the PF-DNA complex was obtained from the intercept-to-slope ratio of $A_0/(A - A_0)$ vs. $1/[\text{DNA}]$ plot. However, it is lower than the binding constant ($1.74 \times 10^4 \text{ M}^{-1}$) of the typical intercalator lumazine-DNA complex in phosphate buffer at pH 7.2.⁵⁷

This indicates that PF binding with DNA has an affinity that is less than the classical intercalator, lumazine, due to its sandwich-like structure.

Voltammetric studies of PF-DNA interactions

For CV titrations, both the concentration and the volume of CK-DNA were kept constant while varying the concentration of PF in the solution. The solutions were deoxygenated *via* purging Ar gas for 10 min before the experiment, and maintaining an Ar atmosphere throughout the measurement.

The diffusion coefficients of PF in both the absence and presence of DNA were determined by using the Randles-Sevcik equation,^{58,59}

Fig. 2 i_p vs. $v^{1/2}$ plots for 6 mM PF in the absence of DNA (●) and the presence of 100 μ M DNA (■) at 20 mV/s (a), 50 mV/s (b), 100 mV/s (c), 200 mV/s (d) and 500 mV/s (e) in 30 mM phosphate buffer (pH 4.7) at 37°C.

$$i_p = 0.4463nFAC_o^*(nF/RT)^{1/2}D_o^{1/2}v^{1/2}, \quad (2)$$

where i_p is the peak current (A), A the surface area (cm^2) of the electrode, C_o^* the bulk concentration (mol cm^{-3}) of the electroactive species, D_o the diffusion coefficient (cm^2/s) and v the scan rate (V/s).

The diffusion coefficients for PF in both the absence and presence of DNA were obtained as 1.34×10^{-10} and $9.54 \times 10^{-11} \text{ m}^2/\text{s}$, respectively, from i_p vs. $v^{1/2}$ plots shown in Fig. 2. It can be seen that the diffusion coefficient of DNA-bound PF is an order of magnitude lower than that of the free PF. Similar results have also been obtained by other investigators.²⁹⁻³¹

In order to explore the binding of PF with CK-DNA, cyclic voltammetric studies were carried out in both the presence and absence of DNA. The peak current (i_p) of the reduction wave of PF was proportional to the square root of the scan rate ($v^{1/2}$). Therefore, the cyclic voltammograms shown in Fig. 3 are diffusion-controlled voltammograms of PF, not the adsorption process of PF on the electrode surface.

The typical CV behavior of 6 mM PF with 0.1 M TBAP as a supporting electrolyte at a glassy carbon electrode in the absence and presence of 300 μ M DNA is shown in Fig. 3. In the presence of DNA, cathodic and anodic peak potentials show 85 and 80 mV shifts to more negative and more positive values, respectively. These shifts in the peak potentials are due to the interaction of both the oxidized and reduced forms with DNA. In the absence of DNA the Fe of PF at the GCE featured reduction of 3+ to 2+ form at a cathodic peak potential of 0.147 V vs. SCE. Its reoxidation to the 2+ form was registered at 0.804 V upon scan reversal. Such a large peak separation of 0.657 V may be attributed to the IR drop effect. The 95 mV difference of E_{pc} and $E_{pc/2}$ indicated the *quasi*-reversibility of the redox process of PF. In the presence of DNA the current decreased sharply as compared to the solution without DNA. This decrease in current is attributed to the decrease in the unbound drug concentration as a consequence of DNA addition due to formation of the PF-DNA complex, while the shift in the peak potentials is attributed to the characteristic behavior of the intercalation of PF into the DNA double-helix.⁶⁰ In the presence of DNA the current is mainly due to unbound species, since the diffusion rate of the DNA-bound species is small.²⁹

The binding parameters were calculated according to the Scatchard equation,⁶¹

$$\gamma/C_u = -K_b\gamma + nK_b, \quad (3)$$

Fig. 3 Cyclic voltammograms of 6 mM PF with 0.1 M TBAP as the supporting electrolyte in the absence (a) and presence (b) of 300 μ M DNA at a 100 mV/s scan rate in a 30 mM phosphate buffer *i.e.* $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ at pH 4.7 and 37°C in 10% aqueous DMF. A glassy carbon electrode (0.071 cm^2) was used as the working electrode and all potentials were reported vs. a saturated calomel electrode (SCE).

Fig. 4 Scatchard plot for the determination of the binding constant and the number of binding sites of PF with 266 μ M DNA in 30 mM phosphate buffer (pH 4.7) at 37°C.

where γ is the ratio of molar concentration of the bound drug to that of the total DNA concentration, C_u the concentration of the unbound drug, K_b the binding constant and n is the number of binding sites on a DNA molecule.

To generate a Scatchard plot, cyclic voltammetric titrations of PF were carried out with DNA. A plot of γ/C_u vs. γ for PF in the presence of 0.266 mM DNA is given in Fig. 4. The binding constant and the number of binding sites were obtained from the slope and the intercept of the Scatchard plot as $3.07 \times 10^2 \text{ M}^{-1}$ and 2.96, respectively, with a good correlation coefficient of 0.9931. The value of the binding constant obtained from Eq. (3) is in close agreement to that obtained from Eq. (1).

For determining the binding site size and the binding constant Carter *et al.* expression¹⁸ was used in the following form:

$$C_b/C_u = K_b[\text{DNA}]/2s - K_bC_b, \quad (4)$$

where C_b is the concentration of the bound drug, C_u the concentration of the unbound drug, K_b the binding constant and s the binding site size per base pair (bp).

A plot of C_b/C_u vs. C_b for PF in the presence of 0.266 mM DNA is given in Fig. 5. The binding constant and the size of the binding site, as obtained from slope, and the slope to the intercept ratio of the Carter *et al.* plot are $3.08 \times 10^2 \text{ M}^{-1}$ and 0.17 bp, respectively. The binding constants obtained from Eqs. (3) and (4) are in very close agreement.

Fig. 5 Carter *et al.* plot for determining the binding constant and the binding-site size of the PF-DNA complex under the conditions mentioned in Fig. 4.

Viscosity measurements

Viscometric titrations were performed by the addition of aliquots of the PF solution into a constant-concentration (200 μM) of DNA solution. Data were presented as η/η_0 vs. varying concentration (ranging from 20 to 80 μM) of PF, where η is the viscosity of DNA in the presence of PF and η_0 is the viscosity of DNA alone.

In addition to CV and UV absorption titrations, viscosity measurements were carried out to provide further clues about the binding mode between PF and DNA. A plot of the relative viscosity (η/η_0) against the concentration of PF in 30 mM phosphate buffer (pH 4.7) at 37°C is shown in Fig. 6. The relative viscosity of DNA shows an increase with the increase in the concentration of PF. In general, a classical intercalation mode causes an increase in the viscosity of DNA solution due to an increased separation of the base pairs at the intercalation sites, and hence an increase in the overall DNA length.⁶² This behavior suggests that PF may bind to DNA *via* intercalation.

The radius (r) of PF in solvated form was calculated from a rearranged form of the Stokes-Einstein equation,

$$r = kT/6\pi\eta D, \quad (5)$$

where η is the viscosity of the solvent, which was determined experimentally, D the diffusion coefficient, which was measured from CV data, T temperature and k Boltzmann's constant.

Whereas the radius of the compound in the solid state was obtained from density measurements, the values of the radii were 7.18×10^{-10} and 9.49×10^{-10} m in the solid state and solution forms. It can be seen that the radius of the compound in the solvated form is greater than that in the solid form, as expected.

Conclusions

The intercalation of PF was investigated by CV, UV-Vis spectroscopy and viscosity measurements. The results show that the intercalation of the drug into DNA takes place. UV-Vis spectrophotometric studies were carried out by keeping the concentration of the drug constant and varying the concentration of DNA. The spectra showed hyperchromism (*i.e.*, increase in absorbance on addition of DNA) and a slight blue shift, which indicated intercalation of the drug into DNA, and causing some unstacking of base pairs. The binding constant, K_b ($3.45 \times 10^2 \text{ M}^{-1}$), measured by UV-Vis absorbance measurements, is lower

Fig. 6 Plot of the relative viscosity (η/η_0) vs. concentration of PF in 30 mM phosphate buffer (pH 4.7) at 37°C.

than the binding constant ($1.74 \times 10^4 \text{ M}^{-1}$) of a typical intercalator, Lumazine.

A change in the voltammetric response of the drug after the addition of DNA was observed in CV. The binding constant, K_b , obtained from Scatchard and Carter *et al.* plots have values of 3.07×10^2 and $3.08 \times 10^2 \text{ M}^{-1}$, respectively, which are in good agreement with the value of K_b obtained from UV-Vis spectroscopy. The number of binding sites in CK-DNA obtained from the Scatchard plot is 2.96, which is comparable with that calculated for telomere DNA⁶³ *i.e.*, 3.2. The size of the binding site (0.17 bp) was obtained from the Carter *et al.* plot.

The diffusion coefficient of PF obtained from a Randles-Sevcik graph was $1.34 \times 10^{-10} \text{ m}^2/\text{s}$, which can be compared with $3.92 \times 10^{-10} \text{ m}^2/\text{s}$ obtained from viscosity measurements using the Stokes-Einstein equation. The decrease in the diffusion coefficient of PF obtained from an i_p vs. $v^{1/2}$ graph, as compared to viscosity measurements, is attributed to an increase in the radius of PF in solution.

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