J. Chem. Thermodynamics 41 (2009) 764–774

Contents lists available at ScienceDirect

J. Chem. Thermodynamics

journal homepage: www.elsevier.com/locate/jct

DNA binding of benzophenanthridine compounds sanguinarine versus ethidium: Comparative binding and thermodynamic profile of intercalation

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ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 4 December 2008
Accepted 6 December 2008
Available online 24 December 2008

Keywords:
Sanguinarine
Ethidium
DNA intercalation
Spectroscopy
Thermodynamics
Drug–DNA interaction

ABSTRACT

There is compelling evidence that cellular DNA is the target of many small molecule anticancer agents. Consequently, elucidation of the molecular nature governing the interaction of small molecules to DNA is paramount to the progression of the rational drug design strategies. In this study, we have compared the binding and thermodynamic aspects of two known DNA binding agents, ethidium and sanguinarine with calf thymus DNA. The study revealed non-cooperative binding phenomena for both the drugs to DNA with an affinity similar for ethidium and sanguinarine as observed from different techniques. The binding phenomena analyzed from isothermal titration calorimetry showed exothermic binding for both compounds that was favoured by negative enthalpy and positive entropy changes typical of intercalative binding. The binding of both the drugs was further characterized by strong stabilization of DNA against thermal strand separation in optical melting as well as differential scanning calorimetry studies. The data of the salt dependence of binding of sanguinarine and ethidium from the plot of $\log K$ versus $\log[Na^+]$ revealed a slope of −0.711 and −0.875, respectively, consistent with the values predicted by the theories for the binding of monovalent cations and the binding free energy has been analyzed for contributions from polyelectrolytic and non-polyelectrolytic forces. The salt dependence of the binding was also evident from the conformational changes in the circular dichroism where both extrinsic and induced changes were lowered on increasing the salt concentration. The heat capacity changes obtained from temperature dependence of enthalpy change gave values of (−590 and −670) $J \cdot mol^{-1} \cdot K^{-1}$, respectively for the binding of sanguinarine and ethidium to DNA. Overall the DNA binding of ethidium was slightly more favoured over sanguinarine.

1. Introduction

DNA interacting agents are of exceptional importance in cancer biology due to their potential therapeutic use. A large number of currently utilized small molecule anticancer agents exert their effect by acting on DNA. For this reason, characterization of the interaction of small molecular ligands with DNA has been the subject of intense investigations for almost half a century [1–5]. Particular importance has been given to natural product small molecules like alkaloids that have potential to form molecular complexes with DNA as they have high abundance and relatively low toxicity. Many natural product small molecules have been proven to be useful as sensitive probes for nucleic acid structures as well [6–8]. Non-covalent interaction of such natural products to DNA mostly involves either an intercalative or minor groove binding mechanism [9]. Most of the planar aromatic natural products bind to DNA essentially by intercalation between the base pairs while crescent shaped molecules bind in the floor of minor groove through H-bonding to the bases. Although no sequence specificity has been clearly delineated in several small molecule interactions, the binding studies continue to build up fundamental data on the molecular nature of various DNA binding aspects hitherto unknown for further development of more effective therapeutic agents.

Sanguinarine and ethidium are polycondensate benzophenanthridine molecules of remarkable biological significance [10–16]. They both have benzophenanthridine chromophore in their structure. Sanguinarine (figure 1a) has methylene dioxy substituents at the 2, 3 and 7, 8 positions while ethidium (figure 1b) has two amino substitutions at the 2 and 8 positions. Both compounds have served as classical models for DNA intercalators for more than four decades. Both have a planar aromatic molecular area, which have been characterized to intercalate between base pairs. Although DNA binding characteristics of these two molecules are known from a vide variety of techniques, there are large discrepancies in the interaction model as well as specificity [17–32]. Structurally, sanguinarine has a pH dependent structural transformation between charged iminium and neutral alkanolamine forms while ethidium has no pH dependent structural change [33]. While sanguinarine has guanine–cytosine (GC) specificity in its DNA binding,

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doi:10.1016/j.jct.2008.12.008

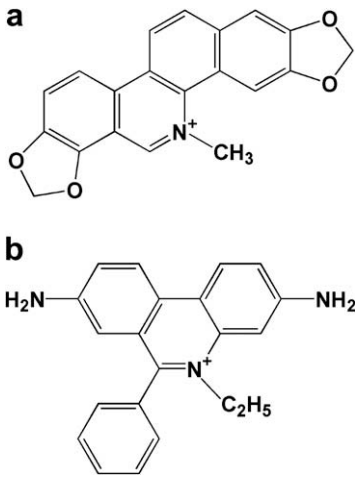

FIGURE 1. Chemical structure of (a) sanguinarine and (b) ethidium.

ethidium is known to exhibit no clear-cut base pair specificity. Knowledge of the complete thermodynamic profiles of DNA interaction of these two molecules will provide information towards understanding the role of molecular forces responsible for the stability of the drug–DNA complexes. We have previously reported the interaction of sanguinarine with several duplex DNA polynucleotides and double stranded poly(A) [31,32]. Studies from our and other laboratories [24–28,31] provided some details on the nature, specificity and energetics of binding but a more detailed analysis, particularly, on the energetic aspects of the interaction seems necessary for a complete understanding of the binding phenomenon. In this study we, therefore, present a complete thermodynamic characterization of the interaction of these two molecules to DNA to correlate the structural data of the intercalation from a comprehensive study using diverse physicochemical techniques.

2. Experimental

2.1. Materials

Calf thymus (CT) DNA was obtained from Sigma–Aldrich Chemicals Co., (St. Louis, MO, USA) and was purified by ethanol precipitation. The ratio of the absorbance at 260 nm to 280 nm was about 1.8 indicating the DNA to be free from protein contamination. Concentration of DNA was determined spectrophotometrically using a molar extinction coefficient (ε) 13,200 $M^{-1} \cdot cm^{-1}$ at 260 nm and expressed in terms of molarity of base pairs throughout. Sanguinarine chloride (SNC) (13-methyl-[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) and ethidium bromide (EB) (3,8-diamino-5-ethyl-6-phenylphenanthridinium) (drugs in general hereafter) were products of Sigma–Aldrich and were used without further purification. Both the drugs were highly soluble in aqueous buffers and hence their solutions were freshly prepared each day and always kept protected in the dark to prevent any light induced changes. Concentrations of these compounds were determined spectrophotometrically at 327 nm for sanguinarine and 480 nm for ethidium. The extinction coefficients (ε) used for sanguinarine and ethidium were of 30,700 $M^{-1} \cdot cm^{-1}$ and 5680 $M^{-1} \cdot cm^{-1}$, respectively. No deviation from Beers law was observed in the concentration range employed in this study.

All experiments were conducted in citrate–phosphate (CP) buffer 10 mM [Na^+], pH 5.5, containing 5 mM Na_2HPO_4. The pH was adjusted by addition of citric acid. Glass distilled deionized water and analytical grade reagents were used throughout. pH measurements were made on Cyberscan 2100 high precision bench pH meter with an accuracy of >±0.001 units (Eutech Instruments Pvt. Ltd., Singapore). All buffer solutions were passed through Millipore filters of 0.45 μm (Millipore India Pvt. Ltd., Bangalore, India) to remove any particulate matter. Salt dependent studies were performed in CP buffer, pH 5.5 containing different amounts of [Na^+].

2.2. Absorbance measurements and analysis of binding data

A Shimadzu Pharmaspec 1700 unit (Shimadzu Corporation, Kyoto, Japan) was used for absorption spectral studies where a constant concentration of the DNA was treated with increasing concentration of the drug in 1 cm path length matched quartz cells with continuous stirring throughout the course of the titration. The amount of free and bound drug was determined as described previously [34,35]. Briefly, following each addition of drug to the DNA solution (40 μM), from the absorbance at the isosbestic point, (353 nm in case of SNC and 512 nm in the case of EB), the total drug concentration (C_t) present was calculated as the ratio of the absorbance at the isosbestic point/extinction at the isosbestic point. This quantity was used to calculate the expected absorbance (A_{exp}) at wavelength maximum of interest (327 nm in the case of SNC and 480 nm in the case of EB), as C_t multiplied by the molar extinction coefficient at the wavelength maximum. The difference in A_{exp} and the observed absorbance (A_{obsd}) was then used to calculate the amount of bound drug as $C_b = A/\Delta\varepsilon = (A_{exp} - A_{obsd})/(\varepsilon_f - \varepsilon_b)$. The amount of free drug was determined by difference, $C_f = C_t - C_b$. The extinction coefficient of the completely bound drug was determined by adding a known quantity of the drug to a large excess of DNA and on the assumption of total binding. Alternatively, the absorbance of a known quantity of the drug was monitored at the wavelength maximum while adding known amounts of DNA until no further change in absorbance was observed. Both these protocols gave identical values within experimental errors in both cases. Binding data obtained from spectrophotometric titration of increasing concentrations of drug to a fixed concentration of DNA were cast into the form of a Scatchard plot of r/C_f *versus* r, where r is the number of ligand (drug) molecules bound per mole of DNA base pairs. Non-linear binding isotherms observed with positive slope at low r values were fitted to a theoretical curve drawn according to the excluded site model of McGhee and von Hippel [36]) for cooperative ligand binding system using the following equation,

$$\frac{r}{C_f} = K \times (1 - \mathrm{nr}) \times \left(\frac{(2\omega - 1)(1 - nr) + (r - R)}{2(\omega - 1)(1 - nr)}\right)^{(n-1)} \left(\frac{1 - (n+1)r + R}{2(1 - nr)}\right)^2, \quad (1)$$

where $R = \{[1 - (n+1)r]^2 + 4\omega r(1 - nr)\}^{\frac{1}{2}}$, and where K is the intrinsic binding constant to an isolated binding site, n is the number of base pairs excluded by the binding of a single ligand molecule, and ω is the cooperativity factor. The binding data were analyzed using the Origin 7.0 software to determine the best-fit parameters of K, n and ω.

2.3. Electrostatic contribution to the binding

The effect of different NaCl concentrations on the binding constant of EB and SNC was determined from the UV absorption studies and this was used to analyze the ionic strength dependence of the equilibrium binding constant according to the polyelectrolytic theory of Record and coworkers [37].

2.4. UV optical melting study

Absorbance versus temperature profiles (melting curves) of DNA and DNA-drug complexes in the range T = (303 to 313) K were