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Interaction of hemoglobin and sulfamethazine: A spectrofluorimetric characterization of the binding thermodynamics

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Studies on the interaction of drugs with bio-macromolecules have been of great interest to understand the molecular aspects of such binding correlating with the structural phenomenon. The binding interaction of a well-known antibiotic drug, Sulfamethazine (SMZ) towards hemoglobin (Hb) have been studied extensively using fluorescence spectroscopic technique. The temperature dependent experiments suggested static quenching and ground state complex formation with the number of binding sites around 1 signifying 1:1 binding ratio with the protein. The thermodynamic parameters obtained from the temperature dependent analyses conveyed entropy driven spontaneous, exothermic reaction. The negative enthalpy and a strong positive entropy contribution suggested dominance of electrostatic force(s) between the protein and the drug. The salt dependent analyses denoted destabilization of the complex with increase in the ionic strength, thus signifying decrease in the electrostatic interaction between Hb and SMZ which is in accordance with the thermodynamic calculations. The partition of free energy change concluded non-polyelectrolytic components to be the dominant factor in the bending between the protein and the drug.

Keywords: Hemoglobin, sulfamethazine, fluorescence, thermodynamics.

Introduction

Drug-protein interaction is an attraction between the drug and the protein influencing the action of drugs. The binding characterizes the action of drug upon a receptor which first needs to bind with the receptor protein or enzyme[1]. The biological characterization of drug-protein interactions has become a demanding issue in pharmaceutical science towards greater interpretation of poly-pharmacology[2]. There are various techniques used for evaluating the drug-protein interactions, among which fluorescence spectroscopy has proved to be one of the best and a very beneficial technique for the study. Fluorescence spectroscopy refers to the absorption of photons in a molecule of shorter wavelength followed by re-emission of the longer wavelength. Due to the absorption, the electrons excites from ground state to higher electronic state[3]. This technique is useful because it involves first singlet excited state of the electrons in the molecule which is sensitive to the local surroundings[4,5]. The efficiency of the quenching technique is used to investigate the binding process to that extent that it records the inclusiveness of the ligand. The binding information from the quenching studies includes strength, thermodynamics, salt dependency, mechanism of binding and characteristics of the protein etc.[6].

Hemoglobin (Hb), an iron containing oxygen transport protein has played a spectacular part in the past account of chemistry, biology and medicine (Fig. 1A). It is found mainly in the red blood cells of all vertebrates and in some inverte-

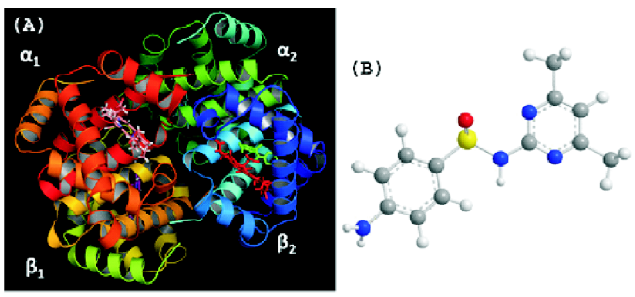

Fig. 1. (A) Structure of hemoglobin (Hb) and (B) structure of sulfamethazine (SMZ) in stick and balls.

brates, RBCs contain maximum amount of Hb[7]. The main function of Hb involves transport of oxygen from the lungs to different tissues of the body at an optimal concentration[8,9]. It also binds with three other gases such as carbon monoxide (CO), carbon dioxide (CO_2) and nitric oxide (NO). The structure of Hb comprises of four subunits, alpha ($\alpha_1$and α_2) chains each with 141 amino acid residues and beta (β_1 and β_2) chains each with 146 amino acid residues, the subunits consist of one polypeptide chain and one heme group respectively[10]. A molecule of Hb can bind with 4 O_2 molecules reversibly following cooperative binding. The binding affinity of O_2 by heme greatly depends on the partial pressure of oxygen (P_{O_2}). In capillary lungs, when the P_{O_2} level is high, around 100 torr and 13.1 kPa the Hb molecule binds with all 4 O_2 molecules whereas with the decrease in the P_{O_2} level (generally when the oxygen demand is high near the muscles) Hb releases its O_2 molecules[10].

Sulfamethazine (SMZ) is a sulphonamide antibacterial veterinary drug with the IUPAC name 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide (Fig. 1B). SMZ inhibits the synthesis of folic acid in bacteria which is an important metabolite for DNA synthesis[11,12]. Veterinary drugs are important portion of domestic industries due to their broad range of advantages that includes diseases treatment, prevention and growth acceleration[13]. SMZ are mostly used to promote growth in cattle and in other veterinary therapeutic purposes, over the last few decade this drug has been detected in the environment from animal excreta in the form of manure, urine etc. in soils and groundwaters[14,15]. In biological research, one of the most important study is the binding of drug molecule with biological molecule because it provides a proper understanding of the interaction which are beneficial in designing, modifying and screening of the drug molecules[16,17]. In this study we have determined the thermodynamic parameters of the interaction between Hb and SMZ through fluorescence quenching study. The data obtained from the thermodynamic studies unravels the molecular characteristics of the interaction along with the corresponding structural details and energetics of the complexation. The thermodynamic data also provides valuable understanding into the pharmacological aspects that determine remedial productiveness in developing novel chemotherapeutics[18].

Materials and methods

Material: Hemoglobin (Hb) and sulfamethazine (SMZ) were procured from Sigma-Aldrich Corporation. Samples preparation and reactions were carried out in citrate phosphate buffer. The [Na^+] was 10 mM of pH 7.0. pH measurements were made on a digital Systronics high precision pH meter (model: MK-VI) with an accuracy of >0.01. All other chemicals and reagents used in this study were of analytical grade obtained from Sigma-Aldrich. Double distilled and deionised water (millipore) was used for the experimental buffer solutions.

Methods:

Fluorescence spectroscopy: Steady state fluorescence and synchronous fluorescence spectra were measured at (25±0.5)°C on an Agilent Cary eclipse spectrofluorophotometer. The fluorescence measurements employed 10 mm optical path length standard quartz cuvettes. For measurements of intrinsic fluorescence of Hb in the presence of SMZ, the protein samples were excited at 295 nm. For the measurement of temperature dependent fluorescence the temperature was maintained at 288.15, 298.15 and 308.15 K and for salt dependent fluorescence study, the concentration of the salt was set at 10, 30 and 100 mM, respectively.

Results and discussion

Temperature dependent fluorescence spectral study:

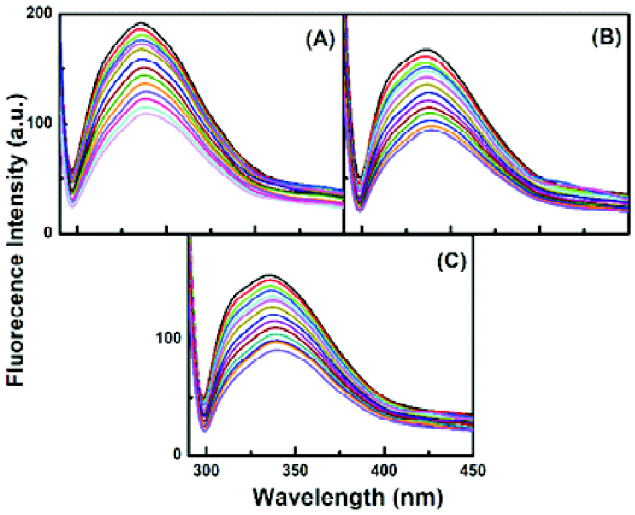

Fig. 2. Spectra of three temperature dependent fluorescence spectroscopy study at (A) 288.15 K, (B) 298.15 K and (C) 308.15 K, respectively.

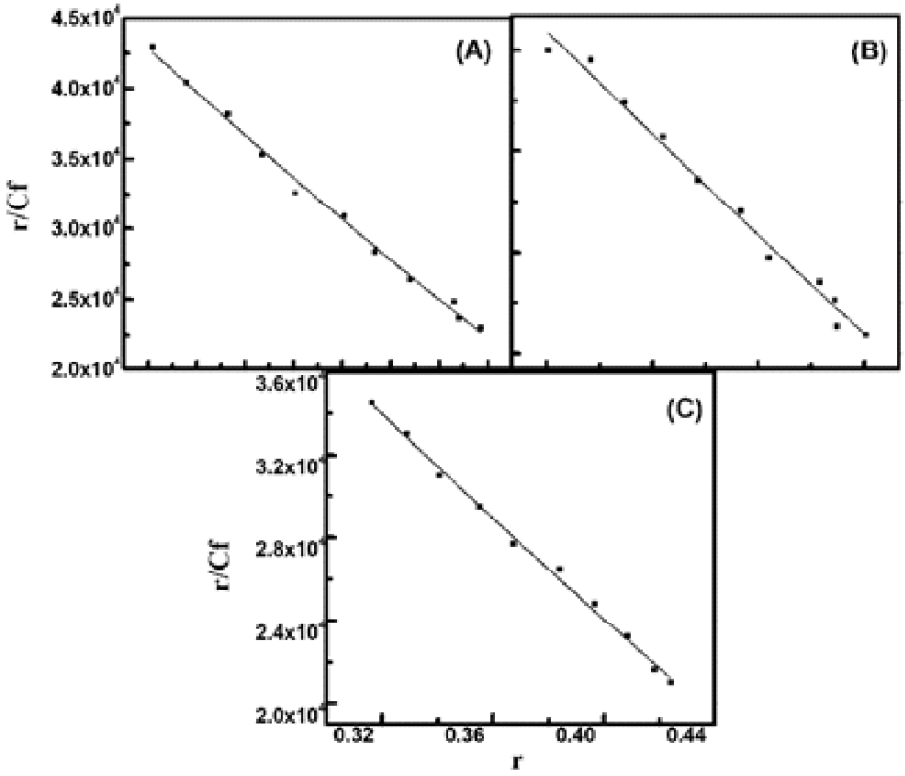

Fig. 3. Scatchard plot for the temperature dependent fluorescence between Hb and SMZ at (A) 288.15 K, (B) 298.15 K and (C) 308.15 K, respectively.

The data obtained from the temperature dependent analysis was done using Scatchard plot (r/C_f versus r, where r is the number of protein molecules bound per drug and C_f is the concentration of the drug SMZ) (Fig. 2)[14,19]. The interaction of SMZ with Hb obtained was found to be nonlinear, so to evaluate the binding parameters with higher accuracy, nonlinear fitting models were used (Fig. 3). The quenching mechanism of the protein molecule may be either due to static or dynamic in nature. Static quenching involves the ground state complex formation while dynamic quenching results from the collision between the protein and the small molecule in the excited state[20]. In order to evaluate the type of quenching pattern, temperature dependent quenching data was analyzed using the nonlinear equation:

$$r/C_f = K \times (1 - n \times x) \times ((1 - n \times x)/(1 - (n - 1) \times x))^{\wedge}(n - 1) \quad (1)$$

where r is the number of protein molecules and C_f is the molar concentration of the free SMZ as mentioned above, n is the no. of protein bound per SMZ and K is the affinity of ligand for the binding site.

Table 1. Binding constant values (K) and number of binding sites (n) for Hb-SMZ interactions at different temperatures

Temp. (K)	$[Na^+]$ (mM)	K (M^{-1})	n
288.15	10	1.0×10^5	1.4
298.15	10	8.1×10^4	1.2
308.15	10	7.9×10^4	1.4

Table 1 show the K values at different temperatures in the order of 10^5–10^4 with n values to be around 1 signifying 1:1 binding ratio between SMZ and Hb. The nonlinear plot at different temperatures – 288.15, 298.15 and 308.15 K respectively indicate only one type of quenching with decrease in the binding affinity. The decrease in the K values on increasing temperature signifies static type of quenching mechanism or ground state complex formation between the protein and the drug.

Binding and thermodynamics of SMZ-Hb from fluorescence spectroscopy:

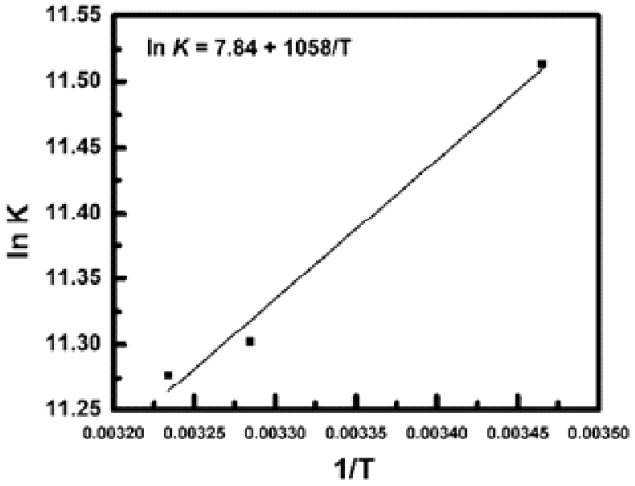

Fig. 4. Van't Hoff plot for Hb-SMZ binding from fluorescence quenching at 10 mM citrate phosphate buffer.

Table 2. Thermodynamic parameters for SMZ-Hb binding from fluorescence quenching at different temperatures

Temp. (K)	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})
288.15	–8.79	18.7	–27.49
298.15		19.3	–28.09
308.15		20.1	–28.89

To understand the thermodynamics of the interaction between Hb and SMZ, detailed thermodynamic data were derived from temperature dependent fluorescence spectroscopy. It provides detailed information on thermodynamic quantities like enthalpy, entropy and also the affinity and stoichiometry of the interaction. The forces involved in the binding interaction of the drug with the protein molecule include non-covalent interactions like hydrophobic interactions, electrostatic, van der Waals and hydrogen bonding. To obtain further insights into the intermolecular interactions, the following thermodynamic parameters like $\Delta G°$, $\Delta H°$ and $\Delta S°$ was determined using the van't Hoff equation[21]:

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad (1)$$

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

where, $\Delta G°$, $\Delta H°$ and $\Delta S°$ are the Gibb's free energy, enthalpy and entropy change. R is the gas constant (R = 8.314×10^{-3} kJ/mol K), T is the temperature (288.15, 298.15 and 308.15 K), and K is the binding constant at the corresponding temperature. Fig. 4 shows the plot of ln K versus 1/T, the slope of which is equal to $-\Delta H/R$ and the intercept give $\Delta S/R$[22]. It is reported that when $\Delta H° < 0$ and $\Delta S° > 0$, the fundamental force is electrostatic force; incase of $\Delta H° < 0$ and $\Delta S° < 0$, it is attributed to van der Waals and hydrogen bond, and when $\Delta H° > 0$ and $\Delta S° > 0$, it is considered as hydrophobic interaction. Table 2 shows the thermodynamic parameters of the interaction, the negative enthalpy of ΔH and a strong positive contribution of ΔS indicates electrostatic interactions in forming SMZ-Hb complex suggesting entropy driven exothermic binding process. The negative free energy change values denotes spontaneity in the binding reaction at all temperature.

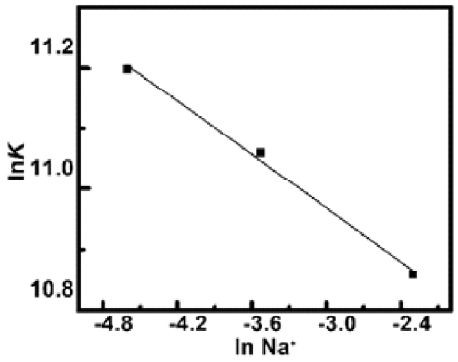

Fig. 5. Van't Hoff plot for Hb-SMZ binding from salt dependent fluorescence quenching at 10, 30 and 100 mM concentrations.

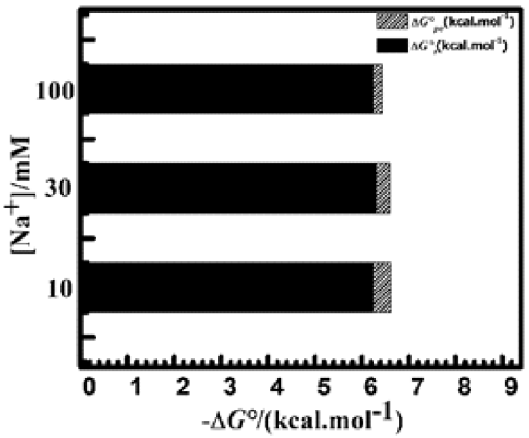

Fig. 6. Non-polectrolytic (black) and poly-electrolytic (shaded) components of binding to Hb and SMZ complex.

Table 3. Salt dependent parameters obtained for Hb-SMZ binding from spectrofluorimetric studies

Complex	$[Na^+]$ (mM)	ΔG° (kcal/mol)	ΔG°_t (kcal/mol)	ΔG°_{pe} (kcal/mol)	$Z\psi$
Hb-SMZ	10	–6.63	–6.25	–0.38	–0.14
	30	–6.60	–6.31	–0.29	
	100	–6.43	–6.24	–0.19	

Salt dependent fluorescence study:

Salt dependent fluorescence experiments were analysed to determine the nature of molecular forces associated in the binding process. The poly-electrolytic contribution to the Gibbs free energy can be determine by partitioning the free energy between poly-electrolytic and non-polyelectrolytic components. So, the experiment was done with respect to different salt concentrations i.e. 10, 30 and 100 mM of $[Na^+]$ concentration, respectively by using fluorescence spectroscopy following Vant't Hoff analysis (Fig. 5).

From poly-electrolytic theory, the slope of the best fit line for a plot of ln *K* versus ln $[Na^+]$ is associated to the counter ion release.

$$\Delta N \text{ (ion)} = \left(\frac{\partial \log K_A}{\partial \log [Na^+]} \right)_{T,P} = -Z\Psi \qquad (3)$$

where ΔN (ion) equivalent to the quantity of counter ions associated upon binding of a drug, *Z* is the apparent charge of the bound ligand and Ψ is the fraction of $[Na^+]$ bound Hb. The low value obtained from the plot determined weak charged interaction between the protein and the drug. Table 3 represents the data obtained from the experiment, it was found that the binding affinity decreases with increase in ionic strength signifying destabilization of the complex with higher salt concentration. The ΔG° was partitioned between electrostatic (ΔG°_{pe}) and non-electrostatic (ΔG°_t) components (Fig. 6). The poly-electrolytic/electrostatic (ΔG°_{pe}) contributions were calculated from the equation:

$$\Delta G^\circ_{pe} = -Z\Psi RT \ln ([Na^+]) \qquad (4)$$

At 10, 30 and 100 mM salt concentrations, the poly-electrolytic (ΔG°_{pe}) components were found to be –0.38, –0.29, –0.19 kcal/mol which are 5.73, 4.3, 2.9% of the total ΔG°, denoting less or weak contribution of charges in the interactions. The non-polyelectrolytic (ΔG°_t) components were analysed from the subtraction of ΔG° and ΔG°_{pe} which were found to be –6.25, –6.31 and –6.24 kcal/mol, respectively. From the higher value of ΔG°_t obtained we can conclude that the role of non-polyelectrolytic interactions dominates the binding process between Hb and SMZ.

Conclusion

This study deals mainly with the thermodynamic parameters between heme protein, Hb and a well-known antibiotic drug, SMZ through fluorescence spectroscopy analyses. The thermodynamic data revealed an exothermic reaction for the binding interaction between the protein and the drug with electrostatic force involved in it. Non-polyelectrolytic components like van der Waal, hydrogen bond, electrostatic and hydrophobic interactions are said to be the dominant factor contributing to the binding which support further to the structural characteristic and energetics of the interactions. The finding helps in the proper understanding of the molecular mechanism quantitatively which can in turn be beneficial for the future pharmacological aspects in discovering novel chemotherapeutics.

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