Structural and Thermodynamic Studies on the Interaction of Iminium and Alkanolamine Forms of Sanguinarine with Hemoglobin

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · MARCH 2014

Impact Factor: 3.3 · DOI: 10.1021/jp409764z · Source: PubMed

2 AUTHORS:

Soumitra Hazra
Indian Institute of Chemical Biology
9 PUBLICATIONS **50** CITATIONS

SEE PROFILE

Gopinatha Suresh Kumar
CSIR-Indian Institute of Chemical Biology
190 PUBLICATIONS **3,349** CITATIONS

SEE PROFILE

THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

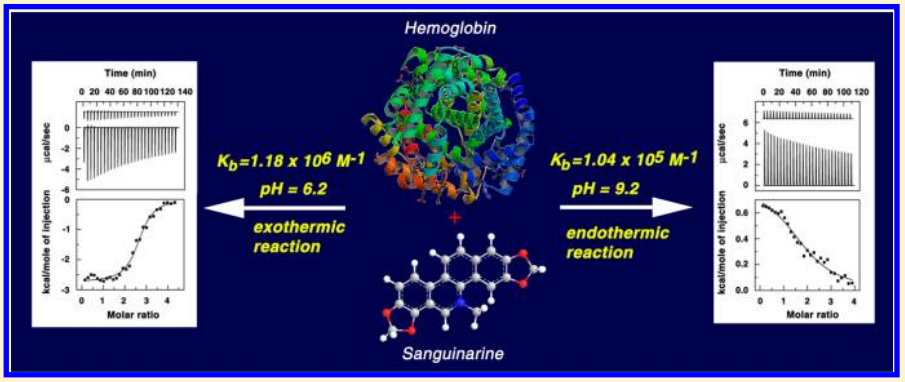
pubs.acs.org/JPCB

Structural and Thermodynamic Studies on the Interaction of Iminium and Alkanolamine Forms of Sanguinarine with Hemoglobin

Soumitra Hazra and Gopinatha Suresh Kumar*

Biophysical Chemistry Laboratory, Chemistry Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700 032, India

Supporting Information



ABSTRACT: Binding of the iminium and alkanolamine forms of the benzophenanthridine anticancer alkaloid sanguinarine to hemoglobin (Hb) was investigated by absorbance, fluorescence, and circular dichroism spectral techniques, and by calorimetry. The binding affinity of the charged iminium was found to be of the order of 10^6 M^{-1}, higher by one order than that of the neutral alkanolamine, from the analysis of the absorbance data. The fluorescence spectral data revealed that the quenching of Hb fluorescence by both forms of sanguinarine is due to the formation of a complex in the ground state and is of an unusual, static nature. Thermodynamic data revealed that the binding of the iminium form was exothermic in nature while that of the alkanolamine was endothermic; the former case predominantly involved electrostatic and hydrogen bonding interactions but the latter was dominated by mostly hydrophobic interactions. Calculation of the molecular distances (r) between the donor (β-Trp37) and acceptor (iminium and alkanolamine) according to Förster's theory suggests both forms of the alkaloid to be bound close to β-Trp37 at the $\alpha1\beta2$ interface of the protein. The iminium form induced greater secondary structural changes in Hb than the alkanolamine as revealed by synchronous fluorescence, circular dichroism and three-dimensional fluorescence spectroscopic studies. These results are consistent with a stronger binding of the iminium over the alkanolamine form. Nevertheless, the hydrophobic probe ANS was displaced from hemoglobin more easily by the alkanolamine form than by the iminium. The study showed that Hb binds more strongly to the biologically active iminium form than the alkanolamine, in contrast to the stronger binding of the latter to plasma protein serum albumin. Overall, this study presents insights on the interaction dynamics and energetics of the binding of the two forms of sanguinarine to hemoglobin.

INTRODUCTION

Binding to proteins is a major factor affecting the absorption, transport, distribution, cellular uptake, and activity of drugs in the circulatory system.[1,2] Carefully studying the binding process by calculating the binding parameters, binding sites, and associated energetics is important for evaluating the pharmacodynamics, pharmacokinetics, distribution, and potential interference leading to effective drug availability, sequestration, etc. Hemoglobin (Hb) is the most important protein in blood cells. It functions in the transport of oxygen from the lungs to different tissues, to disperse hydrogen peroxide, and to effect electron transfer to all body parts and organs. Furthermore, a role of Hb involves transport of H^+, CO_2, and 2,3-bisphosphoglycerate from the tissues to lungs and kidneys. Hemoglobin also carries the important regulatory molecule nitric oxide (NO) bound to the thiol group of globin protein, releasing it along with oxygen.[3] It is the main component of red blood cells (92%), assuming a high concentration of 330 mg/mL under physiological conditions corresponding to a volume fraction (Φ) of 0.25.[4] The high-resolution atomic structure of human Hb revealed[5,6] that it is a tetrameric protein consisting of two identical α-chains and two identical β-chains, each with 141 and 146 amino acid residues, respectively. The α-chains contain seven, while the β-chains display eight helices. The tetrameric conformation of hemoglobin is critical for its biological functions.

Received: October 1, 2013
Revised: March 11, 2014
Published: March 17, 2014

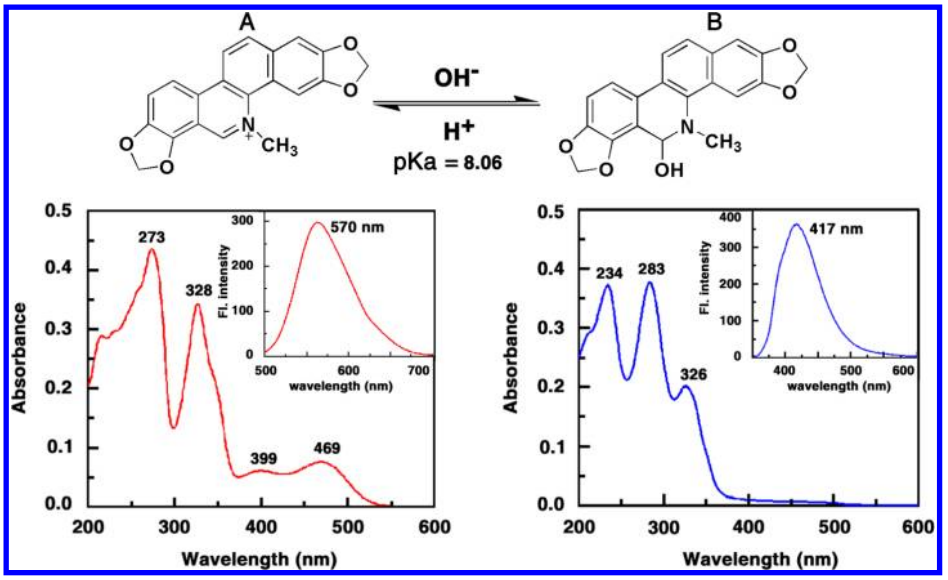


Figure 1. Chemical structure of sanguinarine (A) iminium and (B) alkanolamine forms. Bottom: absorption spectra of iminium and alkanolamine forms (10 μM). Inset shows the fluorescence emission spectra of iminium (10 μM) and alkanolamine (1 μM) forms of sanguinarine.

Sanguinarine (Figure 1) is a quaternary benzophenanthridine alkaloid abundantly found in many botanical species.[7−10] It exhibits various pharmacological effects and is a potential lead compound in cancer therapy inducing apoptosis in a variety of cancer cells through different mechanisms.[11−15] It binds strongly to various DNA and RNA structures like duplex, triplex, and quadruplex, and inhibits the enzyme topoisomerase. The nucleic acid binding properties have been linked to its pronounced anticancer activity.[16,17] It exists as the cationic iminium (Figure 1A) and neutral alkanolamine (Figure 1B) forms with a pK_a of 8.06.[18,19] The charged iminium form has been identified as the nucleic acid binding form.[20,21] It is a potential inhibitor of tumorigenesis and may be a valuable lead compound in the development of more efficient anticancer drugs. Although the DNA and RNA binding properties have been studied in great details,[22−26] binding to proteins is scarcely investigated. We reported the thermodynamics of sanguinarine binding to serum proteins where a stronger binding of the alkanolamine over the iminium form was observed.[27,28] Serum protein, the most abundant protein of blood plasma, essentially transports the exogeneous compounds (drugs or small molecules) while Hb carries mainly oxygen. As the concentration of Hb in the plasma (330 mg/mL) is much higher than that of the albumins (40 mg/mL),[29] it is important not to overlook the interaction of a potential drug candidate with Hb, because this complexation will notably influence the pharmacological action and bioavailability in the human body. Thus, to understand the pharmaceutical utility of sanguinarine and to provide the molecular basis of drug action, its interaction with hemoglobin needed to be clearly understood. The objective of this study was, therefore, to characterize the noncovalent binding phenomena between sanguinarine and hemoglobin from a biophysical perspective. The structural aspects of sanguinarine−Hb interaction and the thermodynamic profile associated with the binding were characterized in detail. The association constant, the binding domain, and the thermodynamic parameters of the complexation were determined through isothermal titration calorimetry (ITC) in addition with multiple spectroscopic techniques and by following the displacement of the hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS). Structural alterations of Hb upon complexation with both forms of sanguinarine were studied by synchronous fluorescence, circular dichroism (CD), and three-dimensional fluorescence techniques. The results of this work, we believe, offer salient clues on the absorption and bioavailability of sanguinarine.

MATERIALS AND METHODS

Materials. Human methemoglobin (H 7379, M = 64 500 Da), sanguinarine (≥98% purity, M = 367.78 Da), and 1,8-anilinonaphthalenesulfonic acid (ANS, ≥97% purity, M = 299.34 Da) were purchased from Sigma−Aldrich. Hemoglobin was further purified by diethyl-aminoethanol (DEAE)-sepharose Fast Flow anion exchange chromatography. The purity of the protein was checked in reversed phase-high performance liquid chromatography and was found to be greater than 99%. The sample was dialyzed into the experimental citrate-phosphate (CP) buffer, 10 mM [Na^+] of pH 6.2 or carbonate−bicarbonate (CB) buffer, 10 mM [Na^+] of pH 9.2. The sample concentrations were determined by using molar extinction coefficient values of 179 mM^{-1} cm^{-1} at 405 nm for hemoglobin,[30] and 30.7 and 21.6 mM^{-1} cm^{-1}, respectively, at 327 nm for the sanguinarine iminium and alkanolamine forms.[31] All other chemicals used were of analytical grade. The buffer solution was prepared from deionized water and passed through membrane filters of pore size 0.22 μm.

Equipments and Measurements. The absorbance spectra were measured at 298.15 ± 1 K on a Jasco V660 double beam double monochromator spectrophotometer (Jasco International Co., Hachioji, Japan) in 1 cm path length quartz cuvettes. Fluorescence spectra were obtained on a Shimadzu RF-5301PC (Shimadzu Corp., Kyoto) fluorescence spectrometer in fluorescence free quartz cuvettes of 1.0 cm path length. The experiments were performed keeping excitation and emission bandwidth of 5 nm. The sample temperature was maintained at 298.15 ± 1 K using an Eyela UniCool U55 water bath (Tokyo Rikakikai Co. Ltd., Tokyo). The fluorescence quenching of the protein was measured using the excitation wavelength of 295 nm for the intrinsic tryptophan fluorophore. Excitation wavelengths were 470 and 327 nm, respectively, for

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

sanguinarine iminium and alkanolamine forms. Temperature dependent fluorescence spectral studies were performed in a Hitachi F4010 unit equipped with a circulating water bath. Synchronous fluorescence spectra were measured in the excitation range of 220−380 nm keeping $\Delta\lambda$ set at 15 and 60 nm. Correction of the fluorescence intensities for the absorption of exciting light and reabsorption of the emitted light was applied to decrease the inner filter effect.

Three-dimensional fluorescence spectroscopy experiments were performed on a PerkinElmer LS55 fluorescence spectrometer (PerkinElmer, Inc., USA). The initial wavelength was set at 200 nm and extended to 340 nm with an increment of 10 nm for each scan. The fluorescence emission spectra of Hb were measured in the wavelength range of 270−500 nm.

Isothermal titration calorimetry experiments were performed in a MicroCal VP-ITC unit (MicroCal Inc., Northampton, MA). The calorimeter was periodically calibrated and verified with dilution experiments described by the manufacturer so that the mean energy per injection was less than 1.30 μcal and standard deviation was below 0.015 μcal. The protocol involved injection of aliquots of degassed Hb solution from the syringe (311 rpm) of the unit into the sample chamber containing the sanguinarine solution (1.4235 mL). Control experiments were performed by injecting identical volumes of Hb solution into the buffer. Each injection generated a heat spike, the intensity of which diminished as the binding progressed and remained constant as the saturation was reached. The area under each heat burst spike was determined by integration using the Origin software to yield the measure of the heat associated with the injection. The heat generated in the control experiments was subtracted from the heat of Hb-sanguinarine reaction to give the heat of the binding. The results were plotted as a function of the molar ratio of Hb/alkaloid, fitted with one set of binding sites model, and analyzed using the software to give the binding affinity (K_b), the stoichiometry (N) and the standard molar enthalpy change of binding (ΔH^o). The standard molar Gibbs energy change (ΔG^o), and the standard molar entropic contribution to the binding ($T\Delta S^o$) were subsequently determined from standard thermodynamic relationships described earlier.[32]

Absorbance vs temperature curves (melting profiles) of Hb and Hb−sanguinarine complexes were obtained on Shimadzu Pharmaspec 1700 unit having a Peltier controlled TMSPC-8 model accessory (Shimadzu Corp., Kyoto) as described in details earlier.[33] In this experiment, the Hb sample (10 μM) was mixed with varying concentrations of sanguinarine in the buffer in the eight-chambered micro optical cuvette (1 cm path length) and the temperature was increased with a heating rate of 0.5 K/min. The absorbance change at 295 nm was continuously monitored. The melting temperature (T_m) was taken as the midpoint of the melting transition as determined from the maxima of the first order derivative plots by the instrument software.

Temperature dependent transitions of the protein as excess heat capacities were measured in a Microcal VP-DSC unit (MicroCal, Inc.) as described previously.[33] The sample and reference cells of the DSC unit were filled with the buffer solution, equilibrated at 303.15 K for 15 min, and scanned to 383.15 K at a scan rate of 60 K/h., repeating until a reproducible baseline was obtained. On the cooling cycle, the sample cell was rinsed, loaded first with the Hb solution and then with Hb-sanguinarine complexes of different molar ratios, and scanned in the same temperature range. Transition temperature (T_m) was taken as at the maximum excess heat capacity. The calorimetric enthalpy (ΔH_{cal}) was obtained from the transition area while the van't Hoff enthalpy (ΔH_v) was obtained by shape analysis of the calorimetric data. The ΔH_{cal} is model-independent and unrelated to the nature of the transition while the ΔH_v is model dependent.

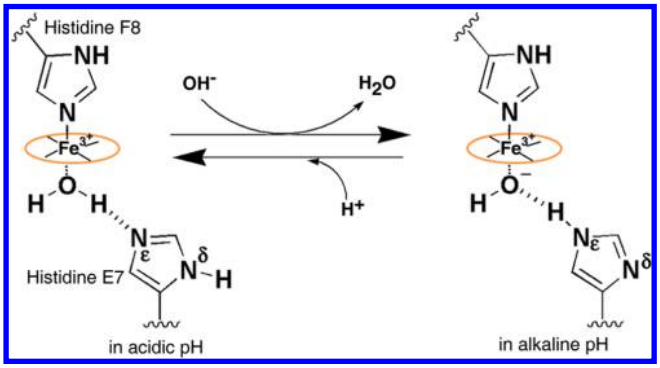
Secondary and tertiary structural changes in the protein on interaction with sanguinarine were measured on a Jasco J815 spectropolarimeter (Jasco International Co.,) in 0.1 and 1 cm cuvettes, respectively, for far UV CD and soret band CD spectra. A Peltier cell holder and temperature controller PFD 425 L/15 were used to maintain the cuvette temperature at 298.15 ± 0.1 K. A scan speed of 20 nm/min., bandwidth of 1.0 nm and sensitivity of 100 milli degrees were applied. Five successive scans were performed and averaged to improve the signal-to-noise ratio and smoothed within permissible limits by the software. The molar ellipticity values were expressed in terms of the mean residue molar ellipticity $[\theta]$, in units of deg cm^2 dmol^{-1}.

RESULTS AND DISCUSSION

Absorption Spectral Studies. Sanguinarine iminium and alkanolamine forms have distinguishable absorption spectral patterns (Figure 1, bottom). In alkaline condition, at pH 9.2, sanguinarine exists as neutral alkanolamine form while at pH 6.2 it is in the charged iminium form.[18,19] The absorption spectrum of Hb at pH 6.2 has two major peaks in the UV−vis region with maxima at 195 nm ($\pi \rightarrow \pi^*$ transition of >C═O group of amino acids) and 406 nm (Soret band), respectively. The heme active sites in methemoglobin exhibits pH-dependent structural change known as "the acid-alkaline transition".[34]

At pH 9.2, the Soret band in hemoglobin is red-shifted from 406 nm and is centered at 413 nm. This is due to conversion of the Fe^{3+}-coordinated ligand H_2O to OH^-, tautomerism of the His E7 imidazole, deprotonation/protonation of His E7 $N_\delta H$ (Scheme 1),[35,36] and consequent changes in the local

Scheme 1. Acid−Alkaline Transition in the Subunit of Methemoglobin



environment around the heme part. The other peak of Hb is blue-shifted to 192 nm. The change in the absorbance spectrum of Hb in the presence of both forms of sanguinarine was monitored to understand the interaction phenomena. In the presence of increasing concentration of sanguinarine, a decrease in the intensity of both bands was observed, the changes being higher with the iminium form. Typical absorbance spectral changes in the Hb spectrum in the presence of iminium and alkanolamine forms of sanguinarine are presented in Figure

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

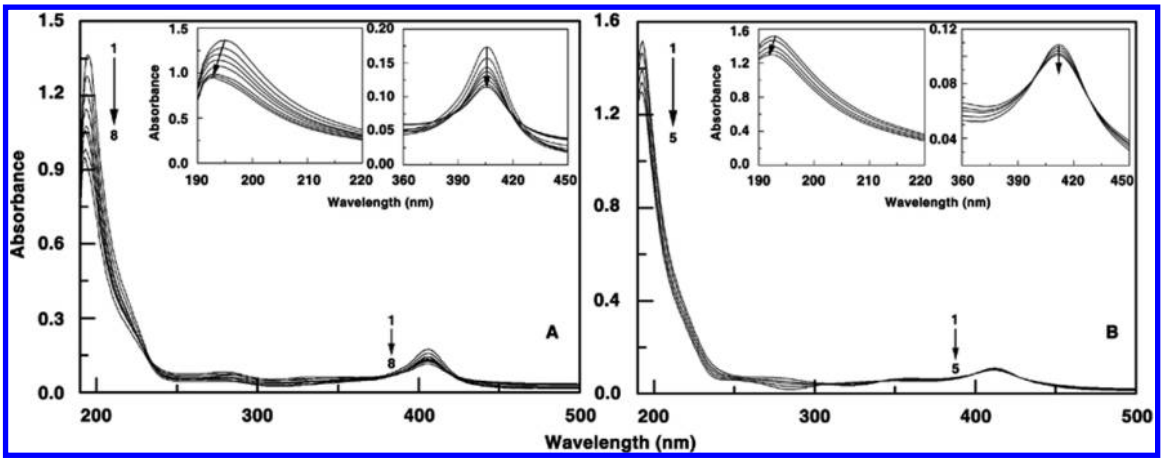


Figure 2. Absorption spectral changes of hemoglobin in presence and absence of sanguinarine. (A) At pH 6.2, hemoglobin (1 μM) treated with 0, 3.9, 7.9, 11.8, 15.7, 19.6, 23.5, and 27.4 μM (curves 1−8) of iminium. (B) At pH 9.2, hemoglobin (1 μM) treated with 0, 3.6, 7.2, 10.8, and 14.4 μM (curves 1−5) of alkanolamine. Inset: 193 and 406 nm bands for Hb-iminium and 192 and 413 nm bands for Hb−alkanolamine interaction are highlighted.

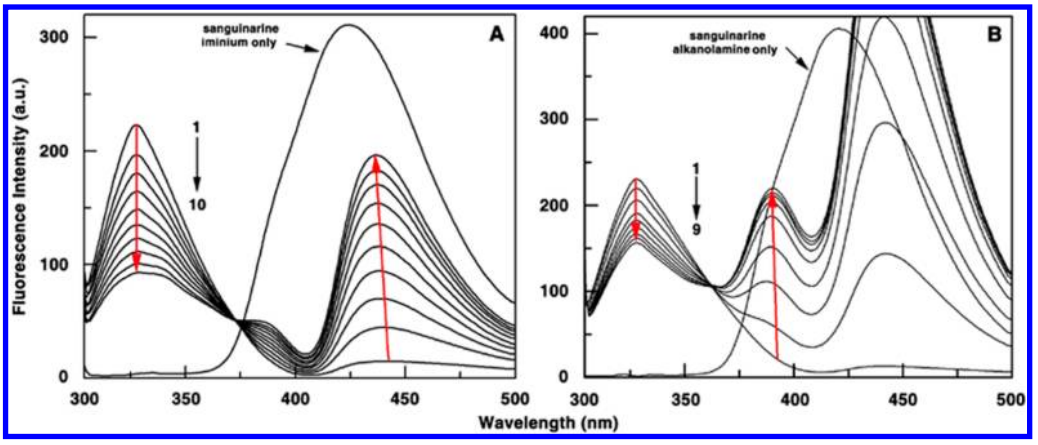


Figure 3. Fluorescence emission spectra of hemoglobin (10 μM) in the presence of various concentrations of sanguinarine (A) iminium and (B) alkanolamine forms. In panel A, curves 1−10 denote 0, 1.5, 2.9, 4.5, 5.9, 7.4, 8.8, 10.2, 11.7, and 13.2 μM of iminium, and in panel B, curves 1−9 denote 0, 3.6, 7.2, 10.8, 14.4, 17.9, 21.4, 24.9, and 28.4 μM of alkanolamine, respectively. The Hb excitation wavelength was 295 nm.

2A,B. In the inset the changes in the 195 and 406 nm bands with the iminium, and in the 192 and 413 nm bands with the alkanolamine forms are showed. In both the cases the effect was hypochromic and somewhat hypsochromic in the former band, the change being more pronounced with the iminium form. The behavior of the Soret band was similar in both the cases and showed only a decrease in the absorbance. This result suggests the formation of a complex at the ground state (static quenching) of both forms of the alkaloid with Hb. The absorbance data in each case were analyzed by the Benesi−Hildebrand equation,[37]

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{1}{K_{BH}(\Delta A_{max})} \times \frac{1}{[Q]} \quad (1)$$

where K_{BH} is the Benesi−Hildebrand binding constant and [Q] is concentration of the free quencher (sanguinarine). Fitting to this equation gave linear plots (Figure S1, Supporting Information) in both the cases with binding affinity values (K_{BH}) of 1.04×10^6 and 1.02×10^5 M^{-1}, respectively, for the iminium and alkanolamine forms. This result suggests that the affinity for Hb is higher with the iminium form than the alkanolamine form.

Fluorescence Spectral Studies. Each $\alpha\beta$ dimer of the tetrameric Hb contains three tryptophan (Trp) residues, namely α-Trp14, β-Trp15 and β-Trp37, yielding a total of six Trp residues.[38] The intrinsic fluorescence of Hb essentially originates from the β-Trp37 at the $\alpha_1\beta_2$ interface,[38] and serves as an indicator for the relaxed form (R) to taut (tense) form (T) transition of the protein. The R form is the oxy (ligand bound) form while the T form is the deoxy form,[39] and they show significant differences in their relative fluorescence intensities. Hb exhibits a fluorescence emission band at 327 nm on excitation at 295 nm, showing that β-Trp37 is buried or in the hydrophobic region.[40] The effect of sanguinarine iminium and alkanolamine forms on the Hb fluorescence intensity is presented in Figure 3, parts A and B. It can be observed that the fluorescence intensity of Hb decreases regularly in the presence of both forms of sanguinarine. This result indicates that both iminium and alkanolamine forms can bind with Hb and quench the intrinsic fluorescence of Hb.

Sanguinarine iminium and alkanolamine forms are good fluorophores; the former emits stronger than the latter. They exhibit emission maxima at 563 and 417 nm, respectively, far away from that of Hb fluorescence maximum. Hence the effect of Hb on the fluorescence of sanguinarine was monitored and

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

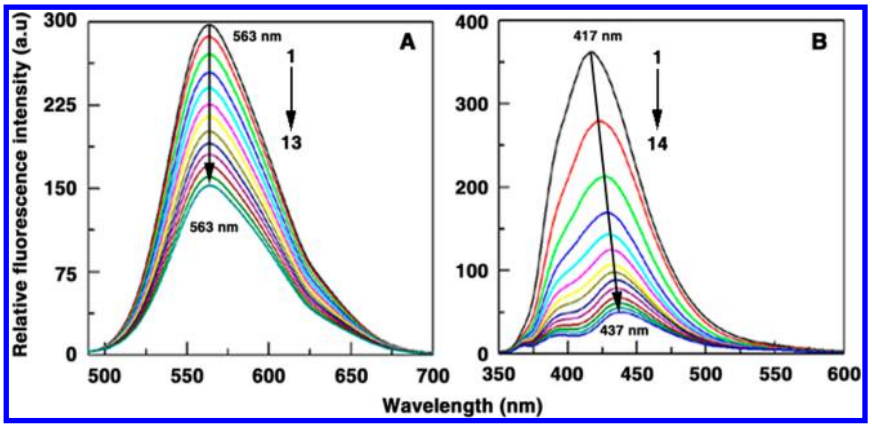


Figure 4. The fluorescence spectra of sanguinarine (A) iminium (10 μM) and (B) alkanolamine (1 μM) forms treated with hemoglobin at pH 6.2 and 9.2, respectively. In panel A, curves 1−13 denote 0, 0.9, 2.8, 4.8, 6.7, 8.6, 10.4, 12.3, 14.2, 16.0, 18.8, 19.7, and 21.6 μM of hemoglobin at pH 6.2, and in panel B, curves 1−14 denote 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 μM of hemoglobin at pH 9.2, respectively. Excitation wavelengths for iminium and alkanolamine were 470 and 327 nm, respectively.

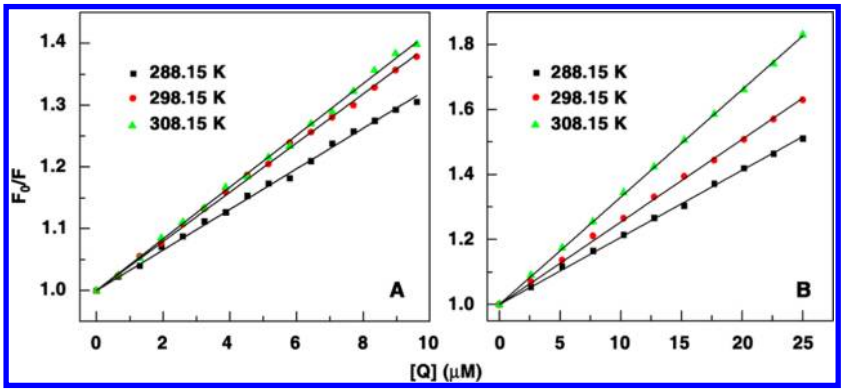


Figure 5. Stern−Volmer plots for the quenching of Hb fluorescence by (A) iminium and (B) alkanolamine forms at different temperatures.

Table 1. Stern−Volmer Quenching Constant Derived for Sanguinarine Binding to Hemoglobin at Different Temperatures[a]

sanguinarine	temp (K)	Stern−Volmer quenching constant (K_{SV}) M^{-1}	quenching rate constant (K_q) M^{-1} s^{-1}	R^b	SD[c]
iminium	288.15	3.281×10^4	3.281×10^{12}	0.9973	0.011
	298.15	3.978×10^4	3.978×10^{12}	0.9987	0.014
	308.15	4.185×10^4	4.185×10^{12}	0.9986	0.021
alkanolamine	288.15	2.064×10^4	2.064×10^{12}	0.9986	0.009
	298.15	2.539×10^4	2.539×10^{12}	0.9988	0.018
	308.15	3.303×10^4	3.303×10^{12}	0.9998	0.025

[a]($\tau_0 = \sim 10^8$ s^{-1}). [b]R is the correlation coefficient for Stern−Volmer plots. [c]SD is the standard deviation for Stern−Volmer plots.

the results are presented in Figure 4, parts A and B. Quenching of the fluorescence intensity of both forms was observed confirming their binding to Hb.

The quenching of the fluorescence of Hb may be either static or dynamic in nature. In order to ascertain the quenching mechanism, temperature dependent fluorescence titration study was carried out and the Stern−Volmer equation was utilized for data analysis.[41]

$$\frac{F_0}{F} = 1 + kq\tau_0[\mathrm{Q}] = 1 + \mathrm{K_{SV}}[\mathrm{Q}] \quad (2)$$

Figure 5 displays Stern−Volmer plots of F_o/F *versus* [Q] at three different temperatures, viz. 288.15, 298.15, and 308.15 K, where F_0 and F denote the steady-state fluorescence intensities of Hb in the absence and presence of quencher (sanguinarine), respectively, K_{SV} is the Stern−Volmer quenching constant, k_q is the bimolecular quenching rate constant, and [Q] is the concentration of the free quencher.

In order to accurately determine the Stern−Volmer quenching constant, the fluorescence emission intensities of Hb and Hb-complexes were corrected for inner filter effect due to the strong absorption of heme protein and sanguinarine in the UV−vis region. The inner filter effect was corrected as described by MacDonald et al.[42]

$$F_{ideal} = F_{obs} \times \mathrm{CF_p} \times \mathrm{CF_s} \quad (3)$$

where F_{ideal} and F_{obs} are the ideal and observed fluorescence intensities, and $\mathrm{CF_p}$ and $\mathrm{CF_s}$ are the correction factors for the primary and secondary inner filter effects, respectively. Lakowicz[43] and others have modified these primary and secondary correction factors for the standard 1 cm square cuvette as $\mathrm{CF_p} \times \mathrm{CF_s} \approx 10^{(A_{ex}+A_{em})/2}$ where A_{ex} and A_{em} are the values of absorbance of the solution at excitation and emission

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

wavelengths (fluorophore and other absorbers). Multiplying this correction factor with the observed fluorescence intensity gives the actual fluorescence emission intensity of the Hb-sanguinarine complexes. Then eq 2 was applied to accurately determine K_{SV} by linear regression of a plot of F_0/F against [Q]. It can be seen that the calculated K_{SV} and k_q values presented in Table 1 increases with increasing temperature for both iminium and alkanolamine forms. This result tempts us to conclude that the quenching mechanism of Hb fluorescence by both forms of sanguinarine is dynamic in nature. Consequently, the fluorescence results contradict the absorption data which was considered as providing evidence for ground state complexation suggesting static quenching. A close look at the values presented in Table 1 reveals k_q to have large enough values, though in dynamic quenching $k_q > 2 \times 10^{10}$ M^{-1} s^{-1} (for dynamic quenching the maximum scattering collisional quenching constant k_q of various quenchers is 2×10^{10} M^{-1} s^{-1}).[41,44−47] To explain this contradictory behavior, Arrhenius' theory was invoked where the rate constant is shown to be dependent on the temperature[47] and the temperature effect on static quenching is more prominent. As per this theory, the viscosity of the solvent decreases with temperature leading to increased chance of collision between sanguinarine molecules and Hb. Hence, dynamic quenching contributes more to the total quenching and K_{SV} decreases as the temperature increases. On the other hand, the value of K_{SV} must increase with increasing temperature according to Arrhenius' theory. If the extent of the increase caused by the rising temperature is larger than the decrease of collision, overall there will be an increase in K_{SV} upon increasing temperature. Thus, the increase in K_{SV} and k_q with temperature suggests a static quenching mechanism, but an unusual one, proving that complex formation is strong for both iminium and alkanolamine forms.

The activation energy of the quenching process can be calculated according to the Arrhenius' equation[48] (eq 4) to evaluate the extent of the temperature impact on the quenching constant,

$$\ln \frac{K_{SV}}{\tau_0} = \ln k_q = -\frac{E_a}{RT} + \ln A \qquad (4)$$

where E_a is the activation energy of the quenching process, and A is the pre-exponential factor. From the slope of ln k_q vs $1/T$ plots, the value of E_a can be determined. A good linear relationship (Figure S2, Supporting Information) indicates that E_a undergoes no significant change in the experimental temperature range. The value of E_a was obtained to be 2.16 and 4.14 kcal/mol, respectively, for the Hb−iminium and Hb−alkanolamine system. It is higher than the E_a values for many other interactions reported earlier[44−46] and this suggests that the effect of temperature on K_{sv} and k_q is much greater in the Hb−alkanolamine system than in the Hb−iminium system.

Thermodynamics of the Binding. The binding of sanguinarine to Hb may essentially involve four types of non covalent forces, viz. electrostatic, hydrophobic, van der Waals, and H-bonding. An idea about the contribution from these forces can be obtained by evaluating the thermodynamic parameters.[49] Therefore, we elucidated the energetics of the interaction from isothermal titration calorimetry (ITC). The thermodynamic parameters like Gibbs energy change ($\Delta G°$), enthalpy of binding ($\Delta H°$), entropy contribution ($T\Delta S°$), binding affinity (K_b), and stoichiometry (N) obtained from ITC may be correlated to the other experiment results. In Figure 6, the representative calorimetric profiles of the titration of the iminium and alkanolamine forms of sanguinarine with

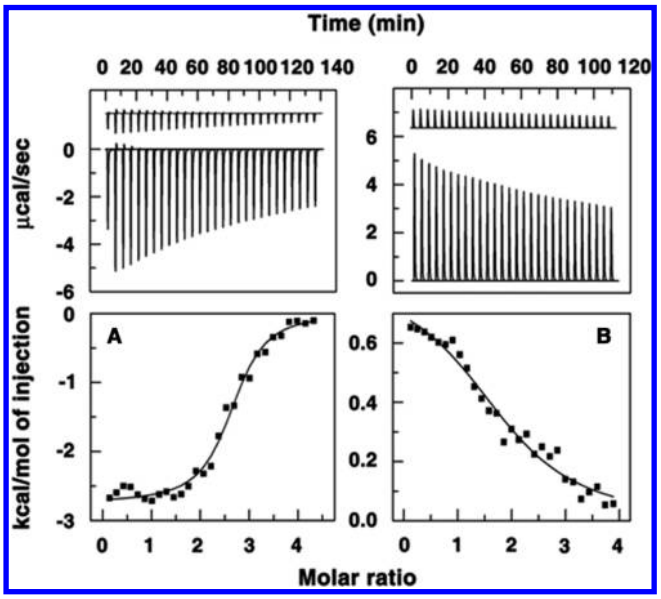


Figure 6. ITC profiles for the binding of sanguinarine to Hb. Top panels present raw results for the sequential injection of hemoglobin of 1000 μM and 900 μM into solutions of (A) iminium (50 μM), and (B) alkanolamine (50 μM) in CP buffer, pH 6.2 and CB buffer, pH 9.2 at 298.15 K, and dilution of Hb into respective buffer (not in scale). The bottom panels show the integrated heat results after correction of heat of dilution against the mole ratio of Hb/sanguinarine. The data points were fitted to one site model and the solid lines represent the best-fit data.

Hb are presented. The binding is an exothermic process for the iminium while it is endothermic for the alkanolamine form. It is a known fact that hydrophobic interactions are low and endothermic, whereas electrostatic interactions are exothermic and higher than the hydrophobic interaction.[50] The isoelectric point of hemoglobin is around 6.8. At pH of 6.2, the protein is essentially neutral in net charge but sanguinarine is positively charged. So electrostatic interactions along with hydrogen bonding interactions will be predominant and the ligand binding will take place via attractive electrostatic interactions between the iminium and the amino acid residues of the protein. A negative value of $\Delta H°$ demonstrates the existence of electrostatic interactions as well as hydrogen-bonding interactions.[51] On the other hand, at pH 9.2, Hb is negatively charged and the interaction may take place predominantly via hydrophobic interactions as sanguinarine is in its neutral form. The endothermic reaction at pH 9.2 indicates hydrophobic interaction playing a major role. There is only one binding event in both the cases. The thermodynamic parameters obtained from the calorimetric studies are summarized in Table 2. The binding constant of iminium at 298.15 K was evaluated to be $(1.18 \pm 0.08) \times 10^6$ M^{-1} while the same for the alkanolamine form was $(1.04 \pm 0.08) \times 10^5$ M^{-1}. The one order higher binding of the iminium over the alkanolamine to Hb was thus evident from the calorimetric data and confirms the results from spectroscopic studies. It is noteworthy to mention that, sanguinarine alkanolamine binds to serum proteins more strongly than iminium and in both cases binding was exothermic.[27,28] Both iminium and alkanolamine forms bind to Hb in a 2:1 ratio as revealed from the N values. The

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

Table 2. Thermodynamic Parameters for the Binding of Sanguinarine with Hemoglobin from ITC Experiments at Different Temperatures[a]

sanguinarine	temp (K)	binding constant, K_b (M^{-1})	stoichiometry (N)	ΔH° (kcal/mol)	$T\Delta S^\circ$ (kcal/mol)	ΔG° (kcal/mol)	ΔC_p° (cal/mol·K)
iminium	288.15	$(9.65 \pm 0.27) \times 10^6$	0.27 ± 0.004	-6.401 ± 0.13	2.805	-3.596 ± 0.13	
	298.15	$(1.18 \pm 0.08) \times 10^6$	0.38 ± 0.004	-7.231 ± 0.98	1.052	-6.108 ± 0.98	−107.6
	308.15	$(1.25 \pm 0.05) \times 10^4$	0.90 ± 0.022	-8.552 ± 1.06	−2.802	-5.750 ± 1.06	
alkanolamine	288.15	$(3.98 \pm 0.33) \times 10^5$	0.44 ± 0.01	3.781 ± 0.09	11.146	-7.365 ± 0.09	
	298.15	$(1.04 \pm 0.08) \times 10^5$	0.51 ± 0.02	1.597 ± 0.06	8.433	-6.836 ± 0.06	−297.6
	308.15	$(7.17 \pm 0.18) \times 10^4$	0.81 ± 0.05	-2.171 ± 0.09	4.682	-6.853 ± 0.09	

[a]All the data in this table are derived from ITC experiments conducted in either 10 mM [Na^+] of CP buffer, pH 7.2 or CB buffer, pH 9.2 and are averages of four determinations. K_b and ΔH° values were determined from ITC profiles fitting to Origin 7.0 software as described in the text.

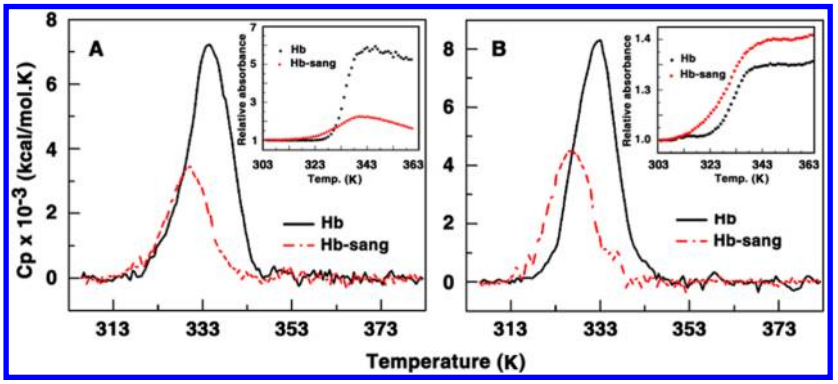


Figure 7. DSC thermograms of (A) Hb (10 μM), Hb−iminium complex (1:5) at pH 6.2, and (B) Hb (10 μM), Hb−alkanolamine complex (1:5) at pH 9.2. In the inset, optical melting profiles for Hb and Hb−sanguinarine complexes.

binding stoichiometry for sanguinarine, around 2.0 for 2:1 binding, was also supported from Job plot data (Figure S3, Supporting Information).

The standard molar Gibbs energy change for the alkanolamine binding at 298.15 K was slightly higher, by about 0.73 kcal/mol, than that of iminium binding. The latter was enthalpy driven with a small favorable entropy change. On the other hand, the former was entropy driven with a small unfavorable enthalpy contribution. The forces driving the interaction between the alkaloid and the protein were examined as a function of temperature (288.15−308.15 K). Overall, with increase in the temperature, the affinity values decreased, and the binding enthalpies became negative with increasing magnitudes for iminium binding. The negative enthalpy of binding at all the temperatures indicated favorable exothermic binding of the iminium form with Hb. With increase of temperature, the entropy contributions decreased but became an unfavorable factor to the binding at 308.15 K. This suggests that the binding is driven by dominant enthalpy contributions for iminium binding to Hb. In the case of alkanolamine binding, the entropy contributions decreased with increasing temperature but remained a favorable factor to the binding even at 308.15 K. Unfavorable enthalpy of binding decreased with temperature and became negative at 308.15 K, i.e., the reaction became exothermic at higher temperature due to the structural reorganization of the protein. It is also evident that binding stoichiometry also changes from two to around one with increasing temperature.

The heat capacity changes (ΔC_p°) accompanying the binding of small molecules to proteins can be determined from the temperature variance of the binding enthalpy.[52] It provides valuable insights into the type and magnitude of binding forces involved in the interaction phenomena. The heat capacity change was obtained by the first derivative of temperature dependence of enthalpy change and the data were plotted as ΔH° versus temperature (Figure S4, Supporting Information). The ΔC_p° values for the binding of iminium and alkanolamine to Hb are −107 and −297 cal/mol·K, respectively. The nonzero and the negative values of ΔC_p° in both the cases indicate that the binding is specific and accompanied by the burial of non polar surface area.[53] The observed enthalpy values varied linearly in the experimental range (288.15−308.15 K) studied (Figure S4, Supporting Information), indicating that there is no measurable shift of the pre-existing equilibrium between the conformational states of the protein in the temperature span studied. A large ΔC_p° value, usually associated with changes in hydrophobic or polar group hydration, is considered to be an indicator of dominant hydrophobic effect in the binding process. The ΔC_p° values fall within the range 100−500 cal/mol·K for a large number of ligand−nucleic acid and ligand−protein interactions.[54,55] The value determined for Hb−iminium interaction is relatively small and negative, whereas that for Hb−alkanolamine interaction is higher in magnitude. The relatively high and negative heat capacity value of Hb-alkanolamine system compared to small value in Hb−iminium system indicates hydrophobic interactions in the protein upon ligand binding; hydrophobic interactions between the ligand and the active site may play a major role at pH 9.2 as discussed earlier also. The difference in the values between iminium and alkanolamine forms indicates the different extent of hydrophobic interaction in these systems. Enthalpy−entropy compensation was observed in both the cases in the temperature range studied.

Analysis of the Destabilization Effects: Differential Scanning Calorimetry and Optical Thermal Melting Studies. Strong binding of small molecules to the proteins usually causes destabilization of protein structures due to the destruction of secondary structure of the protein. This will

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

Table 3. Optical Melting and DSC Data for Sanguinarine Binding to Hemoglobin[a]

pH	system	T_m (K) (optical melting)	T_m (K) (DSC)	ΔH_{cal} (kcal/mol)	ΔH_V (kcal/mol)
6.2	Hb	334.20	334.72 ± 0.05	88.28 ± 0.82	73.34 ± 0.85
	Hb−iminium	328.65	329.86 ± 0.06	40.42 ± 0.45	71.18 ± 1.01
9.2	Hb	331.63	332.14 ± 0.03	96.94 ± 0.65	75.99 ± 0.62
	Hb−alkanolamine	328.42	326.64 ± 0.06	55.39 ± 0.57	70.98 ± 0.90

[a] T_m is the melting temperature. ΔH_{cal} and ΔH_V were obtained from the DSC data.

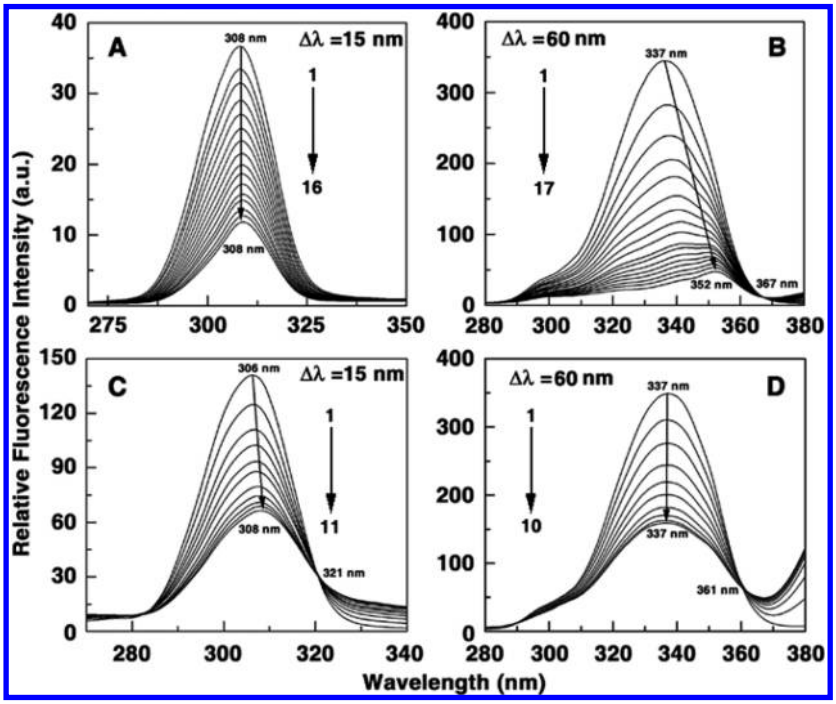


Figure 8. Synchronous fluorescence ($\Delta\lambda$ = 15 and 60 nm) spectra of Hb in the presence of different concentrations of (A, B) iminium and (C, D) alkanolamine, respectively; [Hb] = 10 μM. In panel A, curves 1−16 denote 0 to 21 μM range of iminium; in panel B, curves 1−17 denote 0 to 24 μM range of iminium; in panel C, curves 1−11 denote 0 to 35 μM range of alkanolamine; in panel D, curves 1−10 denote 0 to 35 μM range of alkanolamine concentration.

result in a decrease of the melting temperature (T_m) that can be easily determined from optical melting and differential scanning calorimetry (DSC) experiments. Melting of hemoglobin alone as well as in the presence of sanguinarine was found to be a single transition phenomenon by differential scanning calorimetry (Figure 7). The data derived from DSC and optical melting studies for hemoglobin and hemoglobin-sanguinarine complexes are summarized in Table 3. Melting temperatures of hemoglobin were found to be 334.20 and 331.63 K, respectively, at pH 6.2 and 9.2. In both the cases, Hb was destabilized in the presence of sanguinarine due to protein unfolding upon ligand binding. The ratio between the calorimetric enthalpy (ΔH_{cal}) and the van't Hoff enthalpy (ΔH_v) obtained (Table 3) for the thermal unfolding is not unity, indicating that the melting is not exhibiting a simple two state unfolding behavior. In the presence of both forms of sanguinarine, melting temperature destabilization of about 4−5 K occurred indicating that the binding causes structural alteration in hemoglobin. Similar effect was also observed from optical melting experiments (Figure 7, inset) for both the cases. This destabilization effect on the protein due to the sanguinarine binding was also confirmed by measuring the protein ellipticity change at 222 nm with temperature (Figure S5, Supporting Information).

Energy Transfer from Hemoglobin to Sanguinarine. The Föster resonance energy transfer (FRET) has been used as a "spectroscopic ruler" for measuring molecular distances in biological and macromolecular systems.[56] When the fluorescence emission band of one molecule (donor) overlaps with an excitation band or absorption spectrum of a second (acceptor) molecule, energy transfer takes place and the binding distance between the two molecules can be calculated from FRET experiments.[57,58] Alpert et al.[38] had shown that the intrinsic fluorescence of Hb primarily originates from the β-Trp37 at the $\alpha1\beta2$ interface of the protein. The fluorescence quenching studies presented above confirm that sanguinarine interacts mainly with β-Trp37 of the protein. The overlap of the absorption spectra of sanguinarine iminium and alkanolamine forms with the fluorescence emission spectrum of Hb is shown in Figure S6. According to Förster's theory, the efficiency of energy transfer between the donor and the acceptor (E) can be calculated using the equation

$$E = 1 - \frac{F}{F_O} = \frac{{R_O}^6}{{R_O}^6 + r^6} \quad (5)$$

where r is the distance between the donor and the acceptor, and R_O is the critical distance at 50% energy transfer efficiency. Furthermore

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

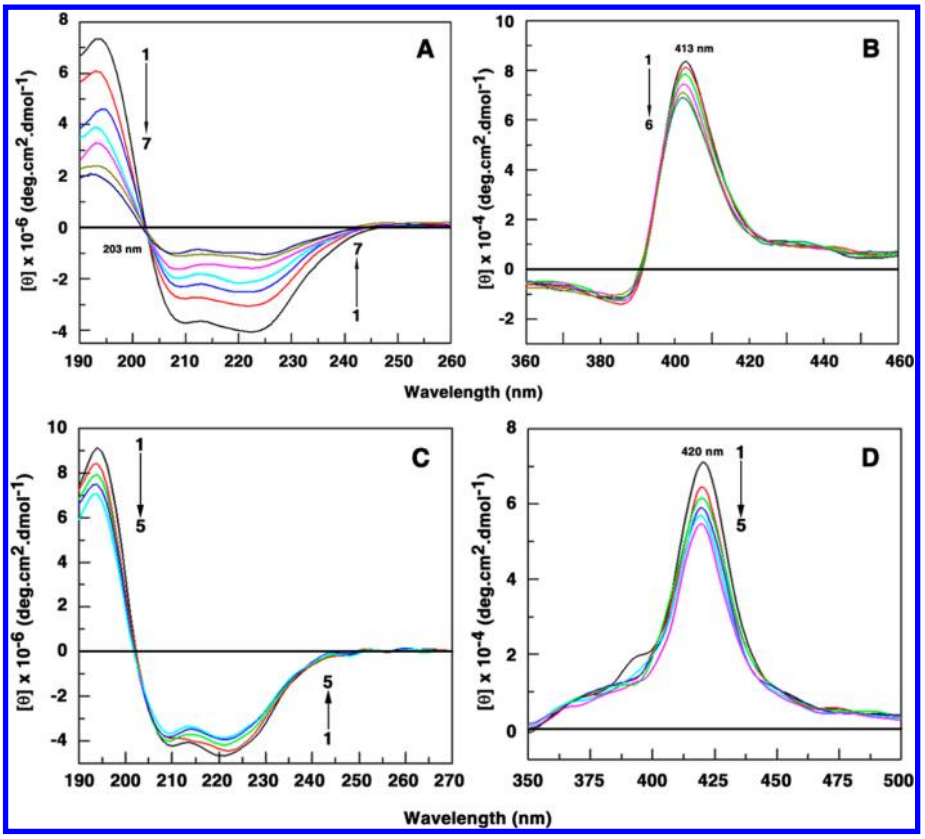


Figure 9. Intrinsic circular dichroic (far UV) spectral changes of Hb (1 μM) in presence of iminium (A) and (C) alkanolamine. In panel A, curves 1−7 denote 0, 1.8, 5.3, 7.0, 8.8, 12.3, and 14.0 μM of iminium, and in panel C, curves 1−5 denote 0, 8.3, 24.9, 41.5, and 58.1 μM of alkanolamine. Soret band CD spectra change of Hb (5 μM) on the interaction with (B) iminium and (D) alkanolamine. In panel B, curves 1−6 denote 0, 1, 2, 3, 5, and 7 μM of iminium, and in panel D, curves 1−5 denote 0, 4.1, 8.2, 12.3, and 16.4 μM of alkanolamine.

$$R_O{}^6 = 8.8 \times 10^{-25} k^2 n^{-4} \varphi J \tag{6}$$

where k^2 is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor, n is the refractive index of the medium, φ is the quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor. The value of J may be calculated by the following equation:

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \tag{7}$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ and $\varepsilon(\lambda)$ is the molar extinction coefficient of the acceptor at λ. The value of J was evaluated by integrating the overlapping region between the donor fluorescence and acceptor absorbance as shown in Figure S6, Supporting Information. In this case, $k^2 = 2/3$, $n = 1.36$ and $\varphi = 0.062$ for Hb.[59] After the correction of inner filter effect for the fluorescence emission intensity for the Hb and Hb-sanguinarine complexes, the values of the parameters found from eq 5−7 were $J = 2.2.96 \times 10^{-14}$ cm^3. L mol^{-1}, $R_0 = 2.499$ nm, $E = 0.263$, and $r = 2.969$ nm for iminium, and $J = 0.709 \times 10^{-14}$ cm^3·L mol^{-1}, $R_0 = 2.055$ nm, $E = 0.088$, and $r = 3.036$ nm for alkanolamine. The donor-to-acceptor distance (r) between sanguinarine and β-Trp37 is smaller than 7 nm, which indicates that energy transfer from Hb to sanguinarine can occur with high probability.[60] It can also be observed that r is very similar for both iminium and alkanolamine forms and hence they bind almost at same distance from β-Trp37 of the protein. This result confirmed again that a static quenching interaction occurs between sanguinarine and Hb according to Förster's non radiative energy transfer theory.

Conformational Changes: Synchronous Fluorescence. Conformational changes during ligand binding to protein can be determined using synchronous fluorescence.[61] According to the theory of Miller,[62] when the difference values between the excitation and emission wavelengths ($\Delta\lambda$) are stabilized at 15 or 60 nm, the synchronous fluorescence spectra of the protein provides characteristic information about Tyr and Trp residues; quenching of protein fluorescence caused by the ligand then implies alteration of the polarity around these amino acid residues. The effect of sanguinarine on the synchronous fluorescence of Hb with $\Delta\lambda$ = 60 nm revealed that the fluorescence intensity diminished systematically with a large red shift of the emission maximum by 15 nm for iminium but almost no shift for alkanolamine binding to Hb (Figure 8). A large red shift is indicative of the change of Trp residues to a more hydrophilic environment and more exposed to solvent compared to that on the binding of the alkanolamine form. Comparatively, there is almost no shift in the maximum emission wavelength using $\Delta\lambda$ = 15 nm for both iminium and alkanolamine forms showing that little transformation takes place in the microenvironment around tyrosines. Therefore, the polarity around β-Trp37 was changed in the presence of the iminium to a large extent but not in the presence of alkanolamine form, while that around Tyr remained unchanged

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

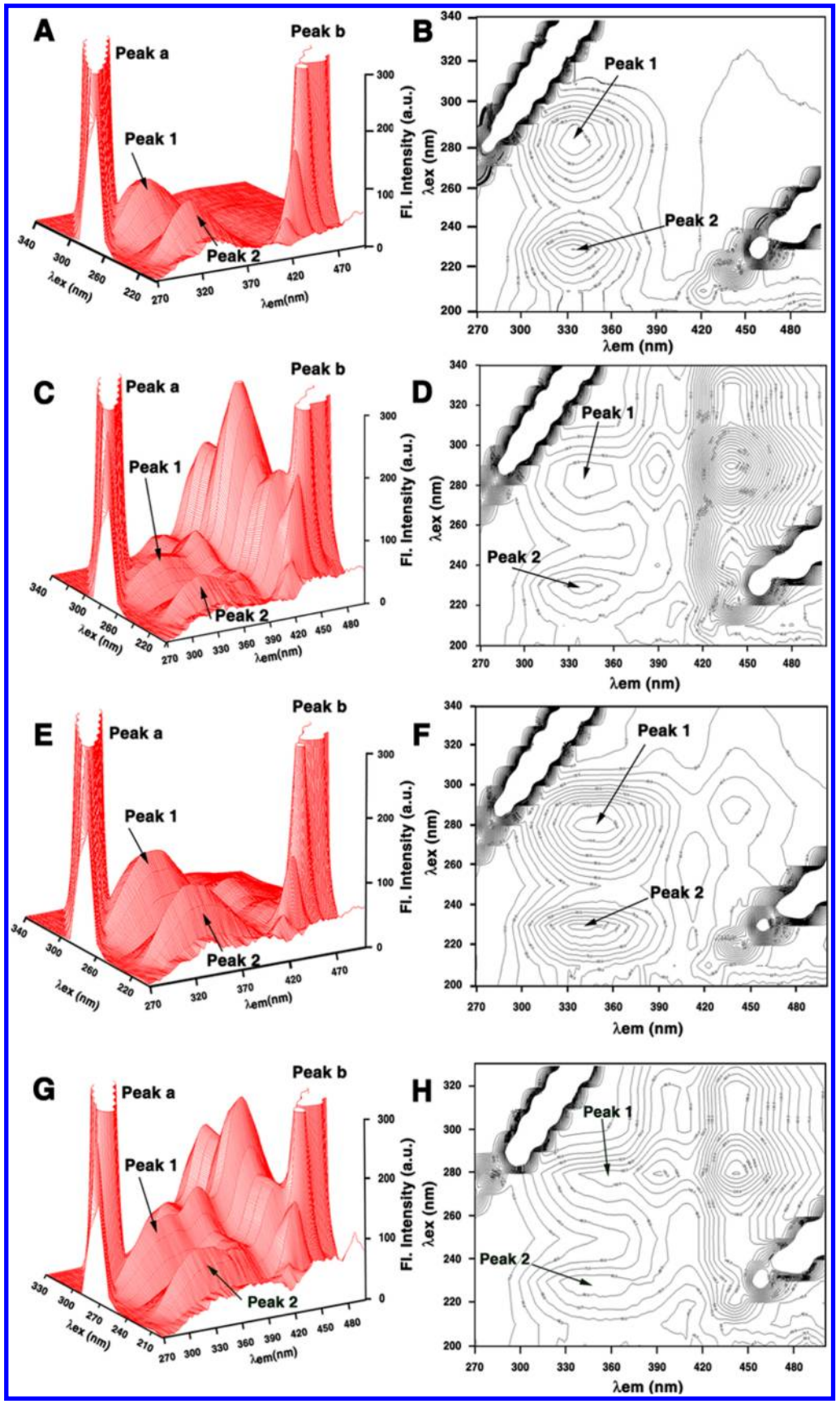


Figure 10. Three-dimensional fluorescence and contour spectra of Hb (A, B), Hb−iminium complex (C, D) at pH 6.2 and Hb (E, F), Hb−alkanolamine (G, H) complex at pH 9.2.

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

Table 4. Data derived from three-dimensional fluorescence of hemoglobin and sanguinarine-hemoglobin interaction

		fluorescence peak 1		fluorescence peak 2	
pH	system	peak position ($\lambda_{ex}/\lambda_{em}$/intensity) (nm/nm/F)	Stokes shift $\Delta\lambda$ (nm)	peak position ($\lambda_{ex}/\lambda_{em}$/intensity) (nm/nm/F)	Stokes shift $\Delta\lambda$ (nm)
6.2	Hb	280/335/100.12	55	230/334/100.82	54
	Hb−iminium	280/339/57.07	59	230/338/65.94	58
9.2	Hb	280/348/127.13	68	230/339/115.07	59
	Hb−alkanolamine	280/358/115.38	78	230/350/103.48	70

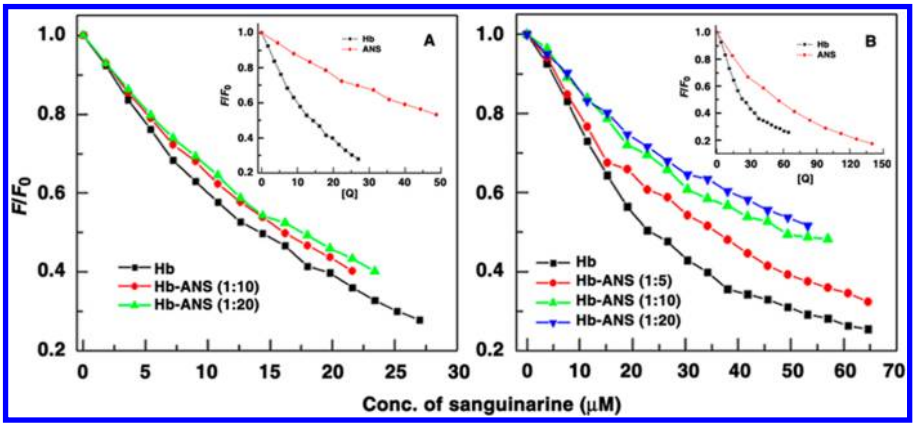


Figure 11. Displacement study of hydrophobic probe ANS bound to Hb by the (A) iminium (pH = 6.2) and (B) alkanolamine (pH = 9.2). Inset: fluorescence quenching profiles of Hb with sanguinarine and ANS.

in both the cases, in agreement with the results from fluorescence quenching and FRET experiments implicating unequivocally the involvement of Trp residue in the binding process.

Circular Dichroism Spectroscopy. Evidence for conformational changes in Hb upon interaction of sanguinarine was obtained from the change in the circular dichroism (CD) spectra. CD is a sensitive technique that can provide information on the conformational changes in a protein on interaction with ligands. Sanguinarine iminium and alkanolamine forms are optically inactive and hence do not have any CD spectra in the entire ultraviolet and visible range. Parts A and C of Figure 9 show that the CD spectrum of Hb exhibits two negative bands in the far-UV region at 208 and 222 nm, respectively; these peaks are characteristic of the α-helical structure of the protein.[63,64] The 208 nm band corresponds to $\pi-\pi^*$ transition of the α-helix and the 222 nm band is due to $n \rightarrow \pi^*$ transition for both the α-helix and random coil; both are contributed by the transition of the peptide bond of the α-helix. The helical content of Hb was calculated according to the equation given in the literature[30,65] and it was found to be 39% and 46%, respectively, in pH 6.2 and 9.2 and close to that reported in earlier observations.[30,66,67] The α-helical content of Hb (1 μM) displayed a reduction from 39% to 3% on binding of the iminium and from 46% to 36% only for alkanolamine form at saturating concentrations of the alkaloid that was 13 and 64 μM, respectively, for the iminium and alkanolamine. Thus, the CD data reveals that the iminium form effected remarkable secondary structural changes in the Hb compared to the alkanolamine, corroborating the results from synchronous fluorescence data. The Soret band CD spectrum of Hb at pH 6.2 has one positive maximum centered at 413 nm and a minimum around 395 nm (Figure 9B). At pH 9.2, the positive maximum of the Soret band CD spectra is shifted to 420 nm due to "the acid-alkaline transition" (Scheme 1)[35,36] and structural changes around the heme part of hemoglobin subunit, whereas the negative minimum band was absent (Figure 9D). The positive maximum was affected in the presence of both forms of sanguinarine. The result indicates structural changes around the heme part of Hb subunit on binding to both forms of sanguinarine.

Three-Dimensional Fluorescence Spectroscopy. This is a new technique that provides evidence for conformational changes in the protein on ligand binding on simultaneous change of excitation and emission wavelengths. The three-dimensional (3D) fluorescence spectra and the contour maps of Hb as well as its complexes with both iminium and alkanolamine forms are shown in Figure 10. The corresponding characteristic parameters are listed in Table 4. In the figure, peak *a* is the first-order Rayleigh scattering peak (λex = λem) and peak *b* is the second-order Rayleigh scattering peak ($\lambda_{em} = 2\lambda_{ex}$). The formation of Hb-sanguinarine complex causes an increase in the diameter of the protein, resulting in an enhanced scattering effect. Peak 1 (λ_{ex} = 280 nm) is primarily the intrinsic fluorescence spectra of Trp and Tyr residues;[68] as the protein excitation is at 280 nm and the fluorescence by the phenylalanine (Phe) residue is negligible here. Beside peak 1, there is another fluorescence peak 2 (λ_{ex} = 230 nm). From the CD spectra (Figure 9), the peaks at 208 and 222 nm, in the far-UV region, are both attributed to the $n \rightarrow \pi^*$ transition for the peptide bond of the α-helix. Therefore, we can infer that peak 2 is mainly caused due to the $n \rightarrow \pi^*$ transition of Hb's characteristic polypeptide backbone. The fluorescence intensities of peaks 1 and 2 decrease in the presence of sanguinarine iminium and alkanolamine forms, but to different extents: the intensity ratios of peak 1 are 1:0.57 and 1:0.90 for the two forms and for peak 2 are 1:0.65 and 1:0.89. The increase of Stokes shift and the decrease of fluorescence intensity of peaks 1 and 2, in combination with the synchronous fluorescence and CD spectral changes, clearly indicate that the binding of sanguinarine to Hb induces an unfolding of the polypeptide chains of Hb, which results a conformational change of Hb

dx.doi.org/10.1021/jp409764z | J. Phys. Chem. B 2014, 118, 3771−3784

yielding an increase of the exposure of some hydrophobic regions that had been buried. Taken together, the binding of sanguinarine to Hb induced secondary structure changes in Hb. The change in Stokes shift value of peaks 1 and 2 due to complex formation is higher in the case of alkanolamine binding indicating that the excited state is more stable in Hb-alkanolamine complex than in the Hb-iminium complex (Table 4).

Hydrophobic Probe ANS Displacement Study. In order to determine the preferred binding region of sanguinarine on Hb, hydrophobic probe displacement study was performed. The fluorescent dye 8-anilino-1-naphthalenesulfonic acid (ANS) is a hydrophobic probe sensitive to microenvironment changes and has often been used to extract information on the hydrophobic binding regions on protein surfaces.[69] It is known that there are two types of binding sites for ANS to the human hemoglobin based on the accessibility to water molecules.[70] The first binding site for ANS is governed mainly by the electrostatic interaction between its sulfonate group and protein cationic groups. The N-terminus of the β-subunit of human hemoglobin forms a cluster of eight positive charges around the central cavity.[71] This allows the ANS molecules to intercalate in this central cavity by electrostatic interaction and be surrounded by a protein globule hydrophobic nonpolar environment.[72] The other binding sites are assigned as localized ANS molecules on the protein segments exposed to the aqueous environment.[72] Both forms of sanguinarine and ANS quench the fluorescence of Hb. However, the extent of quenching by sanguinarine was much higher as compared to ANS (Figure 11, inset). The alkaloid was added to the Hb solution and to mixtures of Hb−ANS complex at 1:5, 1:10, and 1:20 ratios. Plots of the relative fluorescence intensity (F/F_0, where F and F_0 are fluorescence of Hb in the presence and absence of quencher) versus sanguinarine concentration are shown in Figure 11. The results suggest that the alkaloid, when added to the ANS−Hb mixture, can compete with ANS for the hydrophobic regions of the central cavity of the protein. The alkaloid displaces the already bound ANS from the central cavity of Hb and competes with ANS to bind at the hydrophobic region. The displacement of ANS was higher in the case of alkanolamine than iminium due to the neutral nature of the alkanolamine form. The electrostatic interaction of the negatively charged sulfonate group of ANS with the charged iminium form, which is absent in case of its interaction with the alkanolamine, may be the primary reason for the lower displacement of the ANS molecules from the central cavity of Hb. This observation was confirmed by measuring the binding interaction between the sanguinarine iminium and alkanolamine forms with ANS by ITC experiments. It was found that the binding of charged iminium with ANS is 4 fold higher than alkanolamine and the reaction is enthalpically more favorable than that of the alkanolamine (Supporting Information, Table S1). The weaker displacement of ANS by iminium in the Hb−ANS complexes can also be explained by the presence of positively charged group in the ANS binding site. The results revealed that the binding of the alkaloids to Hb was drastically affected by ANS−Hb complex formation that confirms that the alkaloid binds to the hydrophobic region of the Hb central cavity.

CONCLUSIONS

The results presented in this paper reveal that both iminium and alkanolamine forms of the alkaloid sanguinarine bind to hemoglobin. The binding is governed by unusual static quenching mechanism in fluorescence due to ground state complex formation, as revealed by increase in quenching rate constant with temperature. The binding affinity of the charged iminium form was found to be of the order of 10^6 M^{-1}, one order higher than that of the neutral alkanolamine, which is exactly opposite to that was observed in the binding of sanguinarine to serum proteins. As the iminium form is biologically more active at physiological conditions, it will bind more to the hemoglobin than serum protein. The interaction involves close contact with β-Trp37 of the $\alpha1\beta2$ interface as revealed from FRET results. Thermodynamics of the interaction revealed exothermic binding for the iminium and endothermic binding for the alkanolamine as electrostatic interaction plays major role in the binding of the former and hydrophobic interaction in the case of the latter. As revealed from synchronous fluorescence, 3D fluorescence and circular dichroism studies, stronger conformational changes took place in Hb upon binding of the iminium rather than the alkanolamine. The standard central cavity binder 1,8-anilinonaphthalenesulfonic acid affected the binding of alkanolamine and not that of the iminium even though binding distance from the β-Trp37 residue is almost the same for both the forms. This study may provide important biophysical insights on the binding of sanguinarine to hemoglobin.

ASSOCIATED CONTENT

Supporting Information

Figures S1−S6 (Benesi−Hildebrand, Arrhenius, and Job plots and plots of variations of enthalpy versus temperature, changes in ellipticity, and overlap of the Hb fluorescence and absorption spectra) and Table S1 (thermodynamic parameters). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Telephone: +91 33 2472 4049. Fax: +91 33 2473 0284. E-mail: gskumar@iicb.res.in.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the grants from Network Project GenCODE (BSC0123) of the Council of Scientific and Industrial Research (CSIR), Govt. of India. S.H. gratefully acknowledges the CSIR for providing the Senior Research Fellowship. We thank the reviewers for their judicious comments that enabled us to improve the manuscript and Dr. B. Achari, Ex. CSIR-Emeritus Scientist of this institute for the critical reading of the manuscript and valuable suggestions.

REFERENCES

(1) Khazaeinia, T.; Ramsey, A. A.; Tam, Y. K. The Effects of Exercise on the Pharmacokinetics of Drugs. *J. Pharm. Pharm. Sci.* **2000**, *3*, 292−302.

(2) Martinez, M. N.; Amidon, G. L. A Mechanistic Approach to Understanding the Factors Affecting Drug Absorption: A Review of Fundamentals. *J. Clin. Pharmacol.* **2002**, *42*, 620−643.

(3) Connie, C. W.; Hsia, M. D. Respiratory Function of Hemoglobin. *N. Engl. J. Med.* **1998**, *338*, 239−248.

(4) Krueger, S.; Nossal, R. SANS Studies of Interacting Hemoglobin in Intact Erythrocytes. *Biophys. J.* **1988**, *53*, 97−105.

(5) Perutz, M. F.; Rossmann, M. G.; Cullis, A. F.; Muirhead, H.; Will, G.; North, A. C. T. Structure of Haemoglobin: A Three-Dimensional Fourier Synthesis at 5.5-Å. Resolution, Obtained by X-Ray Analysis. *Nature* **1960**, *185*, 416−422.
(6) Perutz, M. F. X-ray Analysis of Hemoglobin. *Science* **1963**, *140*, 863−869.
(7) Facchini, P. J.; Hagel, J. M.; Liscombe, D. K.; Loukanina, N.; MacLeod, B. P.; Samanani, N.; Zulak, K. G. Opium Poppy: Blueprint for an Alkaloid Factory. *Phytochem. Rev.* **2007**, *6*, 97−124.
(8) Abizov, E. A.; Tolkachev, O. N.; Kopylova, I. E.; Luferov, A. N. Distribution of the Sum of Sanguinarine and Chelerythrine in the Above-Ground Part of *Macleaya Microcarpa*. *Pharm. Chem. J.* **2003**, *37*, 413−414.
(9) Vavrečková, C.; Gawlik, I.; Müller, K. Benzophenanthridine Alkaloids of *Chelidonium Majus*; I. Inhibition of 5- and 12-Lipoxygenase by a Non-Redox Mechanism. *Planta Med.* **1996**, *62*, 397−401.
(10) Mahady, G. B.; Beecher, C. W. W. Quercetin-Induced Benzophenanthridine Alkaloid Production in Suspension Cell Cultures of *Sanguinaria canadensis*. *Planta Med.* **1994**, *60*, 553−557.
(11) Han, M. H.; Kim, G.-Y.; Yoo, Y. H.; Choi, Y. H. Sanguinarine Induces Apoptosis in Human Colorectal Cancer HCT-116 Cells Through ROS-Mediated Egr-1 Activation and Mitochondrial Dysfunction. *Toxicol. Lett.* **2013**, *220*, 157−166.
(12) Han, M. H.; Park, C.; Jin, C.-Y.; Kim, G.-Y.; Chang, Y.-C.; Moon, S.-K.; Kim, W.-J.; Choi, Y. H. Apoptosis Induction of Human Bladder Cancer Cells by Sanguinarine Through Reactive Oxygen Species-Mediated Up-Regulation of Early Growth Response Gene-1. *PLoS ONE* **2013**, *8*, e63425.
(13) Lee, J. S.; Jung, W. K.; Jeong, M. H.; Yoon, T. R.; Kim, H. K. Sanguinarine Induces Apoptosis of HT-29 Human Colon Cancer Cells via the Regulation of Bax/Bcl-2 Ratio and Caspase-9-dependent Pathway. *Int. J. Toxicol.* **2012**, *31*, 70−77.
(14) Xu, J.-Y.; Meng, Q.-H.; Chong, Y.; Jiao, Y.; Zhao, L.; Rosen, E. M.; Fan, S. Sanguinarine Inhibits Growth of Human Cervical Cancer Cells Through the Induction of Apoptosis. *Oncol. Rep.* **2012**, *28*, 2264−2270.
(15) Sun, M.; Liu, C.; Nadiminty, N.; Lou, W.; Zhu, Y.; Yang, J.; Evans, C. P.; Zhou, Q.; Gao, A. C. Inhibition of Stat3 Activation by Sanguinarine Suppresses Prostate Cancer Cell Growth and Invasion. *Prostate* **2012**, *72*, 82−89.
(16) Slaninová, I.; Pěnčíková, K.; Urbanová, J.; Slanina, J.; Táborská, E. Antitumour Activities of Sanguinarine and Related Alkaloids. *Phytochem. Rev.* **2014**, *13*, 51−58.
(17) Tsukamoto, H.; Kondo, S.; Mukudai, Y.; Nagumo, T.; Yasuda, A.; Kurihara, Y.; Kamatani, T.; Shintani, S. Evaluation of Anticancer Activities of Benzo[c]phenanthridine Alkaloid Sanguinarine in Oral Squamous Cell Carcinoma Cell Line. *Anticancer Res.* **2011**, *31*, 2841−2846.
(18) Janovska, M.; Kubala, M.; Simanek, V.; Ulrichova, J. Fluorescence of Sanguinarine: Fundamental Characteristics and Analysis of Interconversion Between Various Forms. *Anal. Bioanal. Chem.* **2009**, *395*, 235−240.
(19) Janovska, M.; Kubala, M.; Simanek, V.; Ulrichova, J. Fluorescence of Sanguinarine: Spectral Changes on Interaction with Amino Acids. *Phys. Chem. Chem. Phys.* **2010**, *12*, 11335−11341.
(20) Sen, A.; Maiti, M. Interaction of Sanguinarine Iminium and Alkanolamine Form with Calf Thymus DNA. *Biochem. Pharmacol.* **1994**, *48*, 2097−2102.
(21) Hossain, M.; Kabir, A.; Suresh Kumar, G. Binding of the Anticancer Alkaloid Sanguinarine with tRNA(phe): Spectroscopic and Calorimetric Studies. *J. Biomol. Struct. Dyn.* **2012**, *30*, 223−234.
(22) Maiti, M.; Suresh Kumar, G. Biophysical Aspects and Biological Implications of the Interaction of Benzophenanthridine Alkaloids with DNA. *Biophys. Rev.* **2009**, *1*, 119−129.
(23) Bhadra, K.; Suresh Kumar, G. Therapeutic Potential of Nucleic Acid-Binding Isoquinoline Alkaloids: Binding Aspects and Implications for Drug Design. *Med. Res. Rev.* **2011**, *31*, 821−862.
(24) Bessi, I.; Bazzicalupi, C.; Richter, C.; Jonker, H. R. A.; Saxena, K.; Sissi, C.; Chioccioli, M.; Bianco, S.; Bilia, A. R.; Schwalbe, H.; et al. Spectroscopic, Molecular Modeling, and NMR-Spectroscopic Investigation of the Binding Mode of the Natural Alkaloids Berberine and Sanguinarine to Human Telomeric G-Quadruplex DNA. *ACS Chem. Biol.* **2012**, *7*, 1109−1119.
(25) Giri, P.; Suresh Kumar, G. Molecular Recognition of poly(A) Targeting by Protoberberine Alkaloids: *In Vitro* Biophysical Studies and Biological Perspectives. *Mol. BioSyst.* **2010**, *6*, 81−88.
(26) Roy Chowdhury, S.; Islam, M. M.; Suresh Kumar, G. Binding of the Anticancer Alkaloid Sanguinarine to Double Stranded RNAs: Insights into the Structural and Energetics Aspects. *Mol. BioSyst.* **2010**, *6*, 1265−1276.
(27) Hossain, M.; Khan, A. Y.; Suresh Kumar, G. Interaction of the Anticancer Plant Alkaloid Sanguinarine with Bovine Serum Albumin. *PLoS ONE* **2011**, *6*, e18333.
(28) Hossain, M.; Khan, A. Y.; Suresh Kumar, G. Study on the Thermodynamics of the Binding of Iminium and Alkanolamine Forms of the Anticancer Agent Sanguinarine to Human Serum Albumin. *J. Chem. Thermodyn.* **2012**, *47*, 90−99.
(29) Cox, M. M.; Phillips Jr., G. N. *Handbook of Proteins: Structure, Function and Methods*; John Wiley & Sons Ltd.: West Sussex, U.K., 2007; Vol. 2.
(30) Hazra, S.; Hossain, M.; Suresh Kumar, G. Binding of Isoquinoline Alkaloids Berberine, Palmatine and Coralyne to Hemoglobin: Structural and Thermodynamic Characterization Studies. *Mol. BioSyst.* **2013**, *9*, 143−153.
(31) Jones, R. R.; Harkrader, R. J.; Southard, G. L. The Effect of pH on Sanguinarine Iminium Ion Form. *J. Nat. Prod.* **1986**, *49*, 1109−1111.
(32) Hazra, S.; Hossain, M.; Suresh Kumar, G. Studies on α-, β-, and γ-Cyclodextrin Inclusion Complexes of Isoquinoline Alkaloids Berberine, Palmatine and Coralyne. *J. Incl. Phenom. Macrocycl. Chem.* **2014**, *78*, 311−323.
(33) Islam, M. M.; Sinha, R.; Suresh Kumar, G. RNA Binding Small Molecules: Studies on t-RNA Binding by Cytotoxic Plant Alkaloids Berberine, Palmatine and the Comparison to Ethidium. *Biophys. Chem.* **2007**, *125*, 508−520.
(34) Antonini, E.; Brunori, M. *Hemoglobins and Myoglobins in their Reactions with Ligands*; North Holland Publishing Co.: Amsterdam, 1971; Chapters 2 and 3.
(35) Nagao, S.; Hirai, Y.; Suzuki, A.; Yamamoto, Y. ^{19}F NMR Characterization of the Thermodynamics and Dynamics of the Acid−Alkaline Transition in a Reconstituted Sperm Whale Metmyoglobin. *J. Am. Chem. Soc.* **2005**, *127*, 4146−4147.
(36) Shu, F.; Ramakrishnan, V.; Schoenborn, B. P. Enhanced Visibility of Hydrogen Atoms by Neutron Crystallography on Fully Deuterated Myoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3872−3877.
(37) Benesi, H. A.; Hildebrand, J. H. A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. *J. Am. Chem. Soc.* **1949**, *71*, 2703−2707.
(38) Alpert, B.; Jameson, D. M.; Weber, G. Tryptophan Emission From Human Hemoglobin and its Isolated Subunits. *J. Photochem. Photobiol.* **1980**, *31*, 1−4.
(39) Perutz, M. F.; Fermi, G.; Abraham, D. J.; Poyart, C.; Bursaux, E. Hemoglobin as a Receptor of Drugs and Peptides: X-ray Studies of the Stereochemistry of Binding. *J. Am. Chem. Soc.* **1986**, *108*, 1064−1078.
(40) Burstein, E. A.; Vedenkina, N. S.; Ivkova, M. N. Fluorescence and the Location of Tryptophan Residues in Protein Molecules. *Photochem. Photobiol.* **1973**, *18*, 263−279.
(41) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1999.
(42) MacDonald, B. C.; Lvin, S. J.; Patterson, H. Correction of Fluorescence Inner Filter Effects and the Partitioning of Pyrene to Dissolved Organic Carbon. *Anal. Chim. Acta* **1997**, *338*, 155−162.
(43) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: New York, 2006.

(44) Manna, A.; Chakravorti, S. Role of Block Copolymer-Micelle Nanocomposites in Dye−Bovine Serum Albumin Binding: A Combined Experimental and Molecular Docking Study. *Mol. BioSyst.* **2013**, *9*, 246−257.
(45) Tong, J.-Q.; Tian, F.-F.; Li, Q.; Li, L.-L.; Xiang, C.; Liu, Y.; Dai, J.; Jiang, F.-L. Probing the Adverse Temperature Dependence in the Static Fluorescence Quenching of BSA Induced by a Novel Anticancer Hydrazone. *Photochem. Photobiol. Sci.* **2012**, *11*, 1868−1879.
(46) Tian, F.-F.; Li, J.-H.; Jiang, F.-L.; Han, X.-L.; Xiang, C.; Ge, Y.-S.; Li, L.-L.; Liu, Y. The Adsorption of an Anticancer Hydrazone by Protein: An Unusual Static Quenching Mechanism. *RSC Adv.* **2012**, *2*, 501−513.
(47) Tian, F.-F.; Jiang, F.-L.; Han, X.-L.; Xiang, C.; Ge, Y.-S.; Li, J.-H.; Zhang, Y.; Li, R.; Ding, X.-L.; Liu, Y. Synthesis of a Novel Hydrazone Derivative and Biophysical Studies of its Interactions with Bovine Serum Albumin by Spectroscopic, Electrochemical, and Molecular Docking Methods. *J. Phys. Chem. B* **2010**, *114*, 14842−14853.
(48) Martin, R. S.; Henningsen, R. A.; Suen, A.; Apparsundaram, S.; Leung, B.; Jia, Z.; Kondru, R. K.; Milla, M. E. Kinetic and Thermodynamic Assessment of Binding of Serotonin Transporter Inhibitors. *J. Pharmacol. Exp. Ther.* **2008**, *327*, 991−1000.
(49) Freyer, M. W.; Lewis, E. A. Isothermal Titration Calorimetry: Experimental Design, Data Analysis, and Probing Macromolecule/Ligand Binding and Kinetic Interactions. *Methods Cell Biol.* **2008**, *84*, 79−113.
(50) Landau, M. A.; Markovich, M. N.; Piruzyan, L. A. Studies of the Thermodynamics and Nature of Interaction Between Serum Albumin and Penicillins. *Biochim. Biophys. Acta* **1977**, *493*, 1−9.
(51) Ross, P. D.; Subramanian, S. Thermodynamics of Protein Association Reactions: Forces Contributing to Stability. *Biochemistry* **1981**, *20*, 3096−3102.
(52) Chaires, J. B. Calorimetry and Thermodynamics in Drug Design. *Annu. Rev. Biophys.* **2008**, *37*, 135−151.
(53) Spolar, R. S.; Record, M. T., Jr. Coupling of Local Folding to Site-Specific Binding of Proteins to DNA. *Science* **1994**, *263*, 777−784.
(54) Chaires, J. B. Energetics of Drug-DNA Interactions. *Biopolymers* **1997**, *44*, 201−215.
(55) Guthrie, K. M.; Parenty, A. D.; Smith, L. V.; Cronin, L.; Cooper, A. Microcalorimetry of Interaction of Dihydro-imidazo-phenanthridinium (DIP)-Based Compounds with Duplex DNA. *Biophys. Chem.* **2007**, *126*, 117−123.
(56) Kapanidis, A. N.; Laurence, T. A.; Lee, N. K.; Margeat, E.; Kong, X.; Weiss, S. Alternating-Laser Excitation of Single Molecules. *Acc. Chem. Res.* **2005**, *38*, 523−533.
(57) Stryer, L.; Haugland, R. P. Energy Transfer: A Spectroscopic Ruler. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 719−726.
(58) Valeur, B.; Brochon, J. C. *New Trends in Fluorescence Spectroscopy*; Springer: Berlin, 1999.
(59) Haouz, A.; Mohsni, S. E.; Zentz, C.; Merola, F.; Alpert, B. Heterogeneous Motions Within Human Apohemoglobin. *Eur. J. Biochem.* **1999**, *264*, 250−257.
(60) Valeur, B. *Molecular Fluorescence: Principles and Applications*: Wiley VCH Verlag GmbH: Weinheim, Germany, 2001.
(61) Lloyd, J. B. F. Synchronized Excitation of Fluorescence Emission Spectra. *Nat. Phys. Sci.* **1971**, *231*, 64−65.
(62) Miller, J. N. Recent Advances in Molecular Luminescence Analysis. *Proc. Anal. Div. Chem. Soc.* **1979**, *16*, 203−208.
(63) Woody, R. W. Circular Dichroism. *Method. Enzymol* **1995**, *246*, 34−71.
(64) Woody, R. W. Theory of Circular Dichroism of Proteins, in *Circular Dichroism and the Conformational Analysis of Biomolecules*: Plenum Press: New York, 1996; pp 25−30.
(65) Chen, Y. H.; Yang, J. T.; Martinez, H. M. Determination of the Secondary Structures of Proteins by Circular Dichroism and Optical Rotatory Dispersion. *Biochemistry* **1972**, *11*, 4120−4131.
(66) Mandal, P.; Bardhan, M.; Ganguly, T. A Detailed Spectroscopic Study on the Interaction of Rhodamine 6G with Human Hemoglobin. *J. Photochem. Photobiol. B* **2010**, *99*, 78−86.
(67) Lu, Z.; Zhang, Y.; Liu, H.; Yuan, J.; Zheng, Z.; Zou, G. Transport of a Cancer Chemopreventive Polyphenol, Resveratrol: Interaction with Serum Albumin and Hemoglobin. *J. Fluoresc.* **2007**, *17*, 580−587.
(68) Kang, J.; Liu, Y.; Xie, M.-X.; Li, S.; Jiang, M.; Wang, Y.-D. Interactions of Human Serum Albumin with Chlorogenic Acid and Ferulic Acid. *Biochim. Biophys. Acta* **2004**, *1674*, 205−214.
(69) Stryer, L. The Interaction of a Naphthalene Dye with Apomyoglobin and Apohemoglobin: A fluorescent Probe of Non-polar Binding Sites. *J. Mol. Biol.* **1965**, *13*, 482−495.
(70) Parul, D. A.; Bokut, S. B.; Milyutin, A. A.; Petrov, E. P.; Nemkovich, N. A.; Sobchuk, A. N.; Dzhagarov, B. M. Time-resolved Fluorescence Reveals Two Binding Sites of 1,8-ANS in Intact Human Oxyhemoglobin. *J. Photochem. Photobiol. B* **2000**, *58*, 156−162.
(71) Matulis, D.; Lovrien, R. 1-Anilino-8-Naphthalene Sulfonate Anion-Protein Binding Depends Primarily on Ion Pair Formation. *Biophys. J.* **1998**, *74*, 422−429.
(72) Syakhovich, V. E.; Parul, D. A.; Ruta, E. Y.; Bushuk, B. A.; Bokut, S. B. 1,8-Anilinonaphthalene Sulfonate Binds to Central Cavity of Human Hemoglobin. *Biochem. Biophys. Res. Commun.* **2004**, *317*, 761−767.